

TWO NOVEL MECHANISTIC PARADIGMS IN PIG PREGNANCY:  
I OSTEOPONTIN BINDS TO THE ITGAV (THE A<sub>v</sub> INTEGRIN SUBUNIT) TO  
PROMOTE PORCINE ENDOTHELIAL PROGENITOR CELL INCORPORATION  
INTO DEVELOPING VASCULATURE  
II EXPRESSION AND REGULATION OF GENES FOR GLUCOSE AND ARGININE  
TRANSPORTERS IN PIG UTERI, CONCEPTUSES AND PLACENTAE  
INCREASES DURING PREGNANCY

A Thesis

by

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## ABSTRACT

During pregnancy, uterine and placental blood vessels develop to facilitate maximal transfer of nutrients to the fetus. These studies focus on the incorporation of endothelial progenitor cells (EPCs) into the growing placental and uterine vasculatures (Study 1), and on the expression of the sodium-dependent glucose transporter (*SLC5A1*) and amino acid transporter (*SLC7A3*) in uterine and placental tissues (Study 2). For Study 1, RT-PCR indicated that purified monocytes isolated from newborn piglets differentiated into an endothelial-like phenotype in culture. While human umbilical vein endothelial cells (HUVECs) alone invaded a 3D collagen model to form multicellular tubes with lumens and branching structures, EPCs alone did not invade the matrices. However, when cultured in the presence of HUVECs, EPCs incorporated into vascular structures. Osteopontin (OPN) is a matricellular molecule highly expressed in regions of angiogenesis within the pig placenta. When HUVECs and EPCs were cultured in the presence of OPN, the number of EPCs that incorporated into newly-forming HUVEC sprouts increased significantly. EPCs express integrins, which act as transmembrane receptors for OPN. Affinity chromatography followed by immunoprecipitation studies suggested that the ITGAV subunit on EPCs directly bound OPN. Knocking down the *ITGAV* subunit using siRNA significantly decreased the ability of EPCs to adhere to OPN-coated culture wells in adhesion assays. For Study 2, *in situ* hybridization and qPCR revealed that *SLC5A1* mRNA expression in uterine luminal (LE) and peaked on Day 12-13 of pregnancy. *in situ* hybridization analysis confirmed that *SLC5A1*

expression increased specifically in LE of pseudopregnant model and qPCR revealed that estrogen increased *SLC5A1* mRNA ( $P<0.05$ ) *in vivo* and *in vitro*. *SLC7A3* was induced in the chorion between Days 25 and 30. qPCR confirmed a significant increase in *SLC7A3* mRNA in Day 60 as compared to Day 30 placentae. In conclusion, we hypothesize: (1) that OPN binds to ITGAV-containing integrins on EPCs as an essential initial step in EPC incorporation into the growing vasculature to maximize placental vascularization; and (2) that *SLC5A1* is increased in uterine LE of pigs by estrogens to mediate glucose transport, while *SLC7A3* may mediate arginine transport across the placenta to support developing fetuses in pigs.

## DEDICATION

This thesis is dedicated to my dad, who passed away too early to see me grow up. I have heard stories about you and wish you were still here to see me finish this graduate program. Dad, I am proud to be your son and will always look up to you as a role model. Standing on the stage of 2013 SSR platform competition, the same scenario for which you gave a talk thirty years ago, I felt like we were connected again as father and son.

This thesis is also dedicated to my mom, who raised me up and taught me lessons of life. Mom, thank you for being my biggest fan. You always encourage me when I am down and share my joys together. I cannot express my gratitude to you more.

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## NOMENCLATURE

CAT	Cationic Amino Acid Transporter
DMEM	Dulbecco's Modified Eagle Medium
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EPC	Endothelial Progenitor Cell
FGF	Fibroblast Growth Factor
GE/LE	Glandular Epithelium/ Luminal Epithelium
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GLUT	Facilitated-diffusion Glucose Transporter
HUVEC	Human umbilical Vein Endothelial Cell
ITG	Integrin
OPN	Osteopontin (Secreted phosphoprotein 1 or SPP1)
PFA	Paraformaldehyde
S1P	Sphingosine-1-phosphate
SLC	Solute Carrier Family
siRNA	Small Interfering RNA
SGLT	Sodium-dependent Glucose Transporter
TPA	12-O-tetradecanoylphorbol-13-acetate
Tr	Trophectoderm
VE-CAD	Vascular Endothelia Cadherin
VEGF	Vascular Endothelial Growth Factor

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# CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW

### **Porcine Uterine and Placental Structure**

#### *Uterine Structure*

The uterine wall in pigs differentiates embryologically from the Mullerian ducts into endometrial and myometrial compartments (Mossman 1987). The endometrium consists of a luminal epithelial (LE) layer, secretory glandular epithelial (GE) cells lining the coiling, branching endometrial glands, immune cells, vasculature, and stromal fibroblasts that are spatially arranged into a loose stratum spongiosum and dense stratum compactum (Friess, et al. 1981, Friess, et al. 1982). The myometrium is composed of inner circular and outer longitudinal smooth muscle layers, and vascular elements (Cooke, et al. 1998). Autocrine, paracrine and endocrine communication among these cell types mediate: complex responses to increased uterine blood flow (Ford, et al. 1982); production of and responses to cytokines (Geisert, et al. 1982); changes in water and electrolyte movement (Veldius, et al. 1980); development of and responses to the maternal recognition of pregnancy (Bazer and Thatcher 1977); uterine receptivity to conceptus attachment for implantation (Burghardt, et al. 1997, Keys and King 1980); and uterine secretory activity (Roberts, et al. 1993).

Successful establishment and maintenance of pregnancy requires coordinated communication between the uterus and the conceptus including: (1) secretions from the conceptus to signal pregnancy recognition (Spencer and Bazer 2002) (2) secretions from

the uterine luminal epithelium (LE) and glandular epithelium (GE) i.e., histotroph, to support attachment, development and growth of the conceptus (Ashworth and Bazer 1989, Burton, et al. 2002, Gray, et al. 2001). (3) remodeling at the endometrial LE surface to allow intimate association between conceptus trophoctoderm and endometrium for implantation (Denker 1993, Glasser and Mulholland 1993) and (4) remodeling of the endometrial stroma to generate a cytokine-rich environment that promotes angiogenesis that provides hematotrophic support for the developing conceptus (Dosiou and Giudice 2005, Kliman 2000).

### *Conceptus Elongation*

In pigs (Bazer, et al. 2005), the 1-cell fertilized ovum or zygote undergoes cleavage to form a 2-cell embryo by 26 h after fertilization. Embryos remain in the oviduct before entering the uterus at 48 to 56 h. Blastocyst formation is a critical stage in early embryonic development when cells segregate into the trophoctoderm (Tr), embryonic disc, extra-embryonic endoderm and blastocoel necessary for continued development and differentiation to a conceptus. Before blastocysts develop into a conceptus, they hatch from the zona pellucida. Pig embryos are 0.5 to 1 mm diameter spheres at this stage, and they increase in size through Day 10 of pregnancy (2-6 mm) before undergoing a morphological transition to larger spheres of 10 to 15 mm diameter and then tubular (15 mm by 50mm) and filamentous (1 mm by 100-200 mm) forms by Day 11. Pig conceptuses elongate at 30 to 45 mm/h during the transition from tubular to filamentous forms, primarily by cellular remodeling of trophoctoderm. Porcine

conceptuses subsequently grow to 800 to 1000 mm in length by Day 15 of pregnancy (Bazer 1975). The period of rapid elongation of pig conceptuses is accompanied by estrogen production by Tr (Bazer and Thatcher 1977) along with interferons gamma (IFNG) and delta (IFND) (La Bonnardière, et al. 1991, Lefèvre, et al. 1998).

### *Maternal Recognition of Pregnancy*

The initial conceptus signal to instruct the mother that it is necessary to create an optimal environment for conceptus elongation, implantation and placentation is known as the pregnancy recognition signal. The pregnancy recognition signal in pigs is estrogen, which is secreted by elongating conceptuses on Days 11 and 12 of pregnancy (Bazer 1975, Geisert, et al. 1982, Spencer and Bazer 2004). The importance of estrogen to implantation of pig conceptuses is underscored by the fact that premature exposure of the pregnant uterus to estrogen on Days 9 and 10 results in degeneration of all pig conceptuses by Day 15 (Ashworth, et al. 2006). Estrogen redirects prostaglandin  $F_{2\alpha}$  (PGF) secretion from the uterine vasculature to the uterine lumen, where it is metabolized and thereby sequestered away from the corpora lutea (Bazer and Thatcher 1977, Frank, et al. 1977, Spencer, et al. 2004). This ensures continued production of progesterone, which is required for pregnancy (Bazer 2013). In addition to serving as a pregnancy recognition signal, conceptus estrogens modulate uterine gene expression responsible for endometrial remodeling from Days 13 through 25 of gestation required for implantation (Geisert, et al. 1982) and also correlate with conceptus secretion of interleukin-1 beta, which may be a uterine response to this cytokine (Ross, et al. 2003).

Successful establishment and maintenance of pregnancy requires coordinated communication between the uterus and the conceptus including: (1) secretions from the conceptus to signal pregnancy recognition (Spencer and Bazer 2002) (2) secretions from the uterine luminal epithelium (LE) and glandular epithelium (GE) i.e., histotroph, to support attachment, development and growth of the conceptus; (3) remodeling at the endometrial LE surface to allow intimate association between conceptus trophoctoderm and endometrium for implantation (Denker 1993, Glasser and Mulholland 1993) and (4) remodeling of the endometrial stroma to generate a cytokine-rich environment that promotes angiogenesis that provides hematotrophic support for the developing conceptus (Dosiou and Giudice 2005, Kliman 2000).

### *Implantation*

Implantation is the process by which the blastocyst attaches to the wall of the uterus for juxtaposition of embryonic and maternal circulations leading to the establishment of a functional placenta and successful pregnancy. In pigs, the process is characterized by a lengthy pre-attachment period that includes blastocyst hatching from the zona pellucida and a morphological transition to filamentous conceptus through Days 10 and 12, as well as embryonic migration to produce even spacing within the uterus (Bazer 1975). Attachment of Tr to LE is fully established by Days 12 and 15 of pregnancy (Bazer, et al. 2012b). During the peri-implantation period of pregnancy, LE cells and conceptus trophoctoderm becomes adhesion competent in synchrony to initiate an adhesion cascade within a limited period of time in the uterine wall cycle termed the

“window of receptivity.” These cells demonstrate bi-directional interactions (between the blastocyst and uterine endometrium) involving spatiotemporally regulated endocrine, paracrine and autocrine signals that mediate cell-cell and cell-matrix interactions (Burton, et al. 2002, Choi, et al. 2001, Denker 1993, Hicks, et al. 2003, Lefèvre, et al. 1988, Ott, et al. 1998, Spencer, et al. 2007)

Uterine endometrial responses to implantation are complex. In addition to remodeling of the uterine LE (Aboagye-Mathiesen, et al. 1995), both LE and GE secrete histotroph to nourish and support development of the conceptus (Bebington, et al. 1999). One component of histotroph is osteopontin (OPN), a versatile extracellular matrix protein induced by estrogen in LE where it is hypothesized to influence trophoctoderm and LE adhesion, signal transduction and cell migration (White, et al. 2005). Uterine stromal cell responses include cellular transformation to prevent/control movement of the conceptus through the uterine wall during implantation while generating a cytokine-rich environment that directly promotes angiogenesis to ensure sufficient blood flow to the placenta for hematotrophic nourishment of fetal development (Austin, et al. 2003, Bany and Cross 2006, Li, et al. 2001).

### *Placental Development*

A major function of the placenta is the exchange of gases, micronutrients (including amino acids, glucose) and macromolecules (proteins), as well as the production of hormones, cytokines and other molecules that regulate development of the conceptus throughout gestation (Flint, et al. 1982). In all mammals, the process begins

with attachment of conceptus Tr to the LE (Bazer and First 1983, Joyce, et al. 2008). After successful implantation, the allantois develops outward from the embryo to establish the allantochorion, which determines the size of the functional placenta (Geisert, et al. 1982, Mayhew, et al. 2004, Vallet, et al. 2009). Chorionic trophoblasts adhere to endometrial epithelial cells and the broad extent of the placenta is facilitated by retention of a large allantoic sac. Ultrastructural analyses of the fetal-placental interface have identified interdigitations of microvilli along the surface of the trophoblast and uterine epithelium (Carter and Enders 2013). Pigs have a non-invasive, epitheliochorial placentation as attachment of conceptus to the endometrium remains superficial, and all three maternal and three embryonic cellular layers separating the maternal from fetal circulations remain intact (Friess, et al. 1981, Wooding 1992). Extensive remodeling to form chorionic (placental) ridges and corresponding endometrial intussusception results in folding that maximizes the area of uterine-placental interface. Indentation of the LE and Tr by underlying capillaries further reduces the distance between maternal and fetal blood, providing a short diffusion distance across the placenta (Flint, et al. 1982). This is possibly an advantage for long pregnancies in large animals. It has been debated as to whether this more recently evolved placenta is a more efficient strategy; however, epitheliochorial placentation is optimized to prevent uterine infection or diseases and protects the fetus from the maternal immune system as compared to hemochorial placentas that favor nutrient transport to the fetus through invasion of placental tissues into the uterine wall (Carter and Enders 2013, Huxley 1880, Moffett and Loke 2006, Mossman 1987). The



interdigitation of microvilli between Tr and LE eventually covers the entire placenta except at the openings of uterine glands, where Tr does not attach to LE. These unique, dome-shaped, placental pockets called areolae develop to take up secreted products from endometrial glands that contribute to histotroph that contains proteins, hormones and other nutrients that support fetal growth. A number of the secretory products of the glands including uteroferrin are progesterone-dependent in the pig (Fazleabas, et al. 1985, Simmen, et al. 1991). The transfer of histotroph along with selected molecules from maternal serum may occur by either simple or facilitated diffusion or active transport across the areolae of the chorioallantoic placenta by fluid phase pinocytosis for release into the fetal circulation (Ashworth, et al. 2006, Geisert, et al. 1982). The placenta of each piglet in a litter contains approximately 2,500 areolae and this number is correlated with fetal weight (White, et al. 2005). The fetal nutritional environment regulates circulating levels of important fetal growth factors from the maternal blood to support fetal growth while fetal glucose metabolism is determined by additive effects of fetal plasma glucose and insulin (Bloomfield, et al. 2013b, Hay 2006) Fetal growth and development depends on a maternal supply of nutrients through placental exchange, despite the fact that placenta is a low permeability barrier to nutrient transfer (Smith, et al. 1992). However, transport of the main extracellular ions, such as  $\text{Na}^+$  and  $\text{Cl}^-$ , depends less on diffusion and the transfer rates are consistent with passive transfer (Stulc 1997).

## **Angiogenesis During Pregnancy**

Vasculogenesis is the formation of blood vessels in place by the aggregation of angioblasts into a cord, and is representative of fetal vasculogenesis (Risau, et al. 1988). Postnatal neovascularization is a series of physiological events including proliferation, migration, and remodeling of fully differentiated endothelial cells derived from preexisting blood vessels (Folkman and Shing 1992). In contrast to fetal vasculogenesis, angiogenesis is the formation of new vessels by the sprouting of capillaries from existing vessels (Poole and Coffin 1989). Such development of new capillaries from pre-existing vessels is usually initiated by growth factors, such as bFGF, VEGF, or placental growth factor (PlGF) to initiate mitogenic activation of endothelial cells, induction of serine proteases and collagenases, and increase endothelial chemotaxis and vasodilation (Kaufmann, et al. 2004, Sherer and Abulafia 2001). Pro- and anti-angiogenic factors can directly bind integrin receptors as well to regulate endothelial cell behavior. During angiogenesis, autocrine and paracrine growth pathways regulate integrins on the endothelial surface via tyrosine kinase receptors (Serini, et al. 2008).

The female reproductive system (i.e., ovary, uterus, and placenta) exhibits dynamic, periodic growth and regression in addition to changes in blood flow over the course of each menstrual cycle and over the extent of pregnancy. During gestation, disturbances in uterine blood supply increase risk of perinatal morbidity and mortality caused by preeclampsia, preterm delivery or intrauterine growth restriction. Many diseases such as dysfunctional uterine bleeding, endometriosis, endometrial hyperplasia and/or cancer result from endothelial cell malfunction or remodeling errors due to

deficits or alterations in the expression of angiogenic factors (Zygmunt, et al. 2003). Growth factors are the major regulatory contributors to these physiological events. For example, VEGF and angiopoietin-1 and -2 appear to play major roles in ovarian angiogenesis (Ferrara and Davis-Smyth 1997, Findlay 1986, Jaffe 2000, Reynolds, et al. 1992). An adequate nutrient supply supported by neovascularization is essential for normal fetal development. There is an intimate correlation between the increase of blood flow of the placenta and angiogenesis (Reynolds, et al. 2006). It is clear that neovascularization is critical to conceptus development in all species for maternal-fetal nutrient exchange. Human hemochorial placentation is rich in angiogenic substances that regulate placental vessel formation as well as control maternal vascular adaptation (i.e. vasodilation and vascular permeability) to pregnancy (Zygmunt, et al. 2003). This is also true in porcine epitheliochorial placentation where both uterine and placental layers of epithelium remain intact. For adequate nutrient exchange from maternal to placental vasculatures to be maintained, a tremendous amount of angiogenesis in the uterus and placenta is required (Bazer, et al. 2012b, Sanchis, et al. 2011). Novel *in vitro* culture methods have been used to study angiogenesis. One such system utilizes serum-free media to evaluate capillary morphogenesis during human endothelial cell (EC) invasion of three-dimensional collagen matrices (Davis, et al. 2000). In this model, ECs invade these matrices over a 1–3 day period to form capillary tubes. Improvements in this model system have been useful for elucidating intracellular mechanisms for pro-angiogenic factors that regulate sprout formation. In this improved protocol, endothelial cells are seeded on prepared three-dimensional collagen matrices and rapid invasion into

collagen matrices is driven by sphingosine 1-phosphate (S1P) and growth factors (bFGF and VEGF); endothelial sprouting responses can be quantified and imaged after 24 h (Bayless, et al. 2009). S1P acts through G-protein-coupled receptors (GPCR) to inhibit the Hippo pathway kinases Lats1/2, resulting in stimulation of actin polymerization, and in activation of YAP and TAZ transcription activators, which are oncoproteins repressed by Lats1/2. Hippo pathway may regulate organ size in a tissue-specific manner, but further studies are needed to address these important biological issues. YAP and TAZ are involved S1P-induced gene expression. The pathway may explain endothelial cell and possibly progenitor cell regulation of proliferation and migration to the presence of S1P (Yu, et al. 2012).

### **Endothelial Progenitor Cells**

Endothelial progenitor cells (EPCs) are circulating monocytes that not only express cell surface markers that are similar to those expressed by vascular endothelial cells, but also adhere to endothelium at sites of hypoxia/ischemia. Postnatal bone marrow is known to contain these progenitor cells, and the recruitment of EPCs from the bone marrow into the circulation involves the activation of matrix metalloproteinase-9 (MMP-9), which promotes the transformation of membrane bound Kit-ligand to a soluble Kit-ligand, permitting the transfer of endothelial and hematopoietic stem cells (HSCs) from the quiescent to a proliferative form (Heissig, et al. 2002). EPCs are present in peripheral blood and can proliferate, differentiate, migrate *in situ* and contribute to the process of neovascularization (Asahara 1997). EPCs are characterized

by the expression of cell surface markers, the three most accepted being CD34+, CD133 and the vascular endothelial growth factor receptor-2 (Hristov, et al. 2003). The marker set (CD133+/CD34+/VEGFR-2+/CD14-) represents a small population of progenitor cells with proliferative potential in the circulation, capable of giving rise to late endothelial outgrowth. Cultured EPCs exhibit upregulation of monocyte activation and macrophage differentiation markers (Gulati, et al. 2003, Rehman, et al. 2003). They do not proliferate but secrete potent proangiogenic growth factors (i.e., hepatocyte growth factor, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor) (Hristov and Weber 2004). An important regulator of this EPC mobilization is VEGF, which binds to its receptor VEGFR-2 to mediate further maturation of the cells via the cascade of hemangioblast–angioblast–early EPC–late EPC axis (Hristov, et al. 2003, Hristov and Weber 2004). Recruitment and incorporation of EPCs into sites of neovascularization is a coordinated multistep process including chemoattraction, adhesion, endothelial transmigration, EPC migration, tissue invasion and paracrine factor production (Fusenig and Marmé 2008).

These cells have been isolated and purified from the blood of adult mice, and have been shown to differentiate into endothelial cells *in vitro* (Asahara, et al. 1999). One such method is to isolated monocytes from the blood and then select the progenitor population through selection by outgrowth on fibronectin during *in vitro* culture (Asahara 1997). The resulting cells have been used extensively to determine functional significance. EPC's can mature into the cells that line the lumen of blood vessels (Luttun, et al. 2002, Luttun and Carmeliet 2003). They are important in tumor growth and are

critical for metastasis and angiogenesis (Gao, et al. 2009). Inflammation is an angiogenic process; and the number and function of circulating EPCs changes in many pathological conditions involving inflammation including atherosclerosis, diabetes, and after myocardial infarction. Increased circulating EPCs may account for systemic inflammation along with upregulated levels of growth factors in patients with unstable angina syndromes and no evidence of cardiac necrosis (George, et al. 2004). The functional capabilities of EPCs, such as adhesion to extracellular matrix or migration in response to cytokines, may be as important as the circulating number of cells (Hristov, et al. 2003). Scleroderma patients have a marked reduction in EPCs in the circulation compared with normal healthy controls (Gomer 2008). Improved methods are being developed to quantify EPCs in order to better analyze their role in normal developmental processes as well as vasculopathy (Kuwana and Okazaki 2012, Kuwana, et al. 2004).

The number of EPCs increase during healthy pregnancy and decrease with gestational diabetes (Buemi, et al. 2007). EPC proliferation and number are altered in preeclampsia patients (Matsubara, et al. 2006). In diseases such as endometriosis, up to 37% of the microvascular endothelium of the ectopic endometrial tissue originates from EPCs (Laschke, et al. 2011). Indeed, some recent studies linking EPCs to pregnancy have been published.

### **Osteopontin and Integrins During Pregnancy**

Although originally identified in bone osteoblasts and osteoclasts, hence the prefix “oste”, OPN expression is found in a number of different tissues in many species

that exhibit multiple biological regulatory functions. Similar to in other physiological systems, OPN has been shown to exert multiple functions during pregnancy in mammals (Apparao, et al. 2003, Apparao, et al. 2001, Denhardt and Guo 1993, Johnson, et al. 2003, Liaw, et al. 1995a, Liaw, et al. 1995b, von Wolff, et al. 2001). This extracellular protein is a negatively-charged phosphorylated glycoprotein ligand for several members of the integrin family via an arginine-glycine-aspartic acid (RGD) cell adhesion sequence (Johnson, et al. 2003). Therefore, it can serve as an attachment substrate or linking protein connecting several cell types and hence the suffix "-pontin" (derived from "pons," the Latin word for bridge) (Somerman, et al. 1989). OPN is also known as a marker for the metastatic potential of various cancers as it increases matrix metalloproteases (MMPs) activity along with cell motility, invasion, and angiogenesis (Rangaswami, et al. 2006). In addition, OPN is chemotactic for neutrophils, and this is why OPN plays an important role in many diseases such as neutrophil recruitment in alcoholic liver disease (Apte, et al. 2005, Banerjee, et al. 2006). Integrins are capable of binding to RGD and non-RGD peptide sequences to activate cell signaling pathways. OPN interacts with ITGA4B1 ( $\alpha_4\beta_1$ ) and ITGA9B1 ( $\alpha_9\beta_1$ ) integrin receptors via non-RGD integrin recognition sequences, and interacts several other integrin receptors including ITGA5B1 ( $\alpha_5\beta_1$ ) and ITGAVB1 ( $\alpha_v\beta_1$ ), ITGAVB3 ( $\alpha_v\beta_3$ ), ITGAVB5 ( $\alpha_v\beta_5$ ), ITGAVB6 ( $\alpha_v\beta_6$ ), ITGAVB8 ( $\alpha_v\beta_8$ ) and ITGA8B1 ( $\alpha_8\beta_1$ ) via its RGD domain (Johnson, et al. 2003).

Most cells adhere to OPN via the ITGAVB3 and ITGAVB5 which are integrins known to activate tumor-induced angiogenesis (Hu, et al. 1995b, Liaw, et al. 1995b,

Somerman, et al. 1989). Integrins binding to the RGD site are known to enhance cell adhesion (Hu, et al. 1995a, Liaw, et al. 1994, Ross, et al. 1993), but the potential physiological role of integrins binding to non-RGD sites are not fully understood (Katagiri, et al. 1996, Nasu, et al. 1995, Smith, et al. 1996, Van Dijk, et al. 1993). In pigs, OPN mRNA and protein are expressed in the luminal and glandular epithelium of the endometrium as well as in select mesenchymal cells within the stroma of both the placenta and uterus, although the temporal and spatial expression may vary slightly between different breeds such as Meishans and Large Whites (Garlow, et al. 2002, Hernandez, et al. 2013, White, et al. 2005). OPN expression has been correlated with litter size, conceptus growth and has been reported to improve development of porcine embryos *in vitro* (Garlow, et al. 2002, Hao, et al. 2008, Korwin-Kossakowska, et al. 2001). There is considerable literature supporting the physiological role of OPN in embryo implantation, placentation, and placental development (Garlow, et al. 2002, Johnson, et al. 2003, Johnson, et al. 1999a, Johnson, et al. 1999b, Nomura, et al. 1988, White, et al. 2005). In sheep, progesterone and interferon-tau (the pregnancy recognition signal) induce expression, secretion and proteolytic cleavage of OPN by uterine epithelium (Bailey, et al. 2010, Garlow, et al. 2002, Johnson, et al. 1999b, White, et al. 2005). Additionally in a mouse model, OPN and ITGB3 (the  $\beta_3$  integrin subunit) are co-expressed and are used as biological markers for endometrial receptivity because both play critical roles in blastocyst implantation (Liu, et al. 2013). In humans, OPN was found to be associated with endometrial maturation (Bhagwat, et al. 2013) and is upregulated during the receptive phase of the uterus as well (Bhagwat, et al. 2013).



Integrins are heterodimeric transmembrane receptors consisting of non-covalently linked  $\alpha$  (alpha) and  $\beta$  (beta) chains that are capable of binding extracellular matrix and counter receptors on cells. More than one integrin receptor can recognize a specific ligand and more than one ligand can bind a specific integrin receptor (Humphries 2006). The receptor dimer is largely extracellular; and both subunits terminate in short cytoplasmic domains. There are 19 alpha and 8 beta subunit genes in mammals that can form 25 different receptors (Campbell and Humphries 2011, Humphries 2006, Humphries 2000). Integrins were first identified as integral, transmembrane protein complexes which mediate divalent cation-dependent cell-cell and cell-matrix adhesion (Kuwana and Okazaki 2012, Tamkun, et al. 1986, Xiong, et al. 2001). The cytoplasmic domains are necessary for integrin-mediated signal transduction and the connection with the actin cytoskeleton. The head domain of the focal adhesion protein talin binds to integrins to form a link to the actin cytoskeleton via two actin binding sites in the rod domain (Calderwood, et al. 1999). During pregnancy in mice and other mammalian species, integrins are dynamically expressed and regulated within the uterus and placenta. Trophoblast adhesion and migration at the onset of implantation in rodents are due to changes in expression or distribution of integrin receptors (Sutherland, et al. 1993). Receptors for fibronectin (ITGA5B1), laminin (ITGA6B1) ( $\alpha_6\beta_1$ ) and vitronectin (ITGAVB3) are expressed continuously throughout the peri-implantation period while the expression of other integrin subunits ITGA1 ( $\alpha_1$ ), ITGA2 ( $\alpha_2$ ), ITGA3 ( $\alpha_3$ ) and ITGA7 ( $\alpha_7$ ) subunits are developmentally regulated during trophoblast invasion (Sutherland, et al. 1993). Other integrin subunits (ITGA6, ITGA7) ( $\alpha_6$ ,  $\alpha_7$ ) that can form

receptors for laminin are detected only in the differentiating invasive trophoblast (Sutherland, et al. 1993). Integrins bind to various extracellular matrix components and cell adhesion molecules to transduce cellular signals in uterine epithelial cells and conceptus trophoctoderm (Burghardt, et al. 2002, Giancotti and Ruoslahti 1999, Hynes 1992). The number of potential integrin heterodimers and the number and diversity of ligands that interact with these integrins, ranging from classical ECM molecules to growth factor binding proteins to peptides, accounts for the importance of these receptors in the process of epitheliochorial implantation. Due to the non-invasive nature of placentation in domestic species, integrins are likely to participate in the mechanical coupling of the ECM with the cytoskeleton in uterine LE and conceptus Tr and transmittal of signals to affect processes such as conceptus elongation and cellular differentiation of cells at the maternal-fetal interface (Burghardt, et al. 2002). In addition to roles in implantation or cell attachment to the ECM, integrins play an important role in triggering cell signaling cascades in most cell types including EPCs. Integrin ITGA5B1, ITGA6B1, ITGAVB3 and ITGAVB5 are involved in EPC homing, invasion, differentiation and paracrine factor production while ITGB2s (the  $\beta_2$  integrin subunit) are the major regulator of EPC transendothelial migration (Caiado and Dias 2012). Chapter II will present data indicating a role for ITGAVs, which are known to promote actin polymerization (Zaidel-Bar 2013). The importance of ITGAV subunit during implantation is also underscored in the rabbit model where several strategies used to inhibit the function of the ITGAVB3 reduced implantation sites (Illera, et al. 2003). In human, altered expression of ITGAVB3 correlates with several pathological conditions

associated with infertility (Lessey, et al. 1992). Furthermore, paracrine factor production by EPCs is also regulated by the integrin-ECM interactions since EPCs plated on different matrices (e.g. gelatin, fibronectin, or fibrin) produce different level of paracrine factors (e.g. IL-16, platelet derived growth factor-BB, hepatocyte growth factor (HGF), etc.) (Barsotti, et al. 2011).

### **Glucose and Its Transporters**

The oxidation of glucose represents a major source of metabolic energy in mammalian species, but the plasma membrane is impermeable to polar molecules such as glucose. Therefore, cellular uptake of important nutrients relies heavily on membrane-associated carrier proteins that bind and transfer them across the plasma membrane (Banerjee, et al. 2009). The Human Genome Organization (HUGO) Nomenclature Committee Database has organized a list of transporter families of the solute carrier (*SLC*) gene series to help understanding of the huge family of carrier proteins, including glucose and amino acid transporters. It includes 43 families and 298 transporter genes (Hediger, et al. 2004). Notably, The SLC2 family of glucose transporters comprises 13 members, including the facilitated-diffusion glucose transporters (GLUT) 1-12 and five of these GLUTs (GLUT1 through GLUT4 and GLUT7) have been identified in mammals (Uldry and Thorens 2004). The terms *SLC* and GLUTs are used interchangeably in the literature. The former represents the gene name and the latter represents a descriptive protein name. In sheep and other ruminants (goats, cows, and red deer), GLUT1 was localized to the basal membranes of trophoblast and uterine

epithelium while GLUT3 was found at the microvillous junctions between epithelial and trophoblast cells of sheep (Wooding, et al. 2005). Also in sheep, the *SLC2A1* and *SLC5A1* mRNAs and proteins were abundant in uterine luminal epithelia and superficial glandular epithelia (LE/sGE), while *SLC2A4* was present in stromal cells and glandular epithelia (GE). *SLC2A3* mRNA was not detectable in endometria. *SLC2A1*, *SLC2A3*, *SLC2A4*, and *SLC5A1* are all glucose transporters that were expressed in the trophectoderm and endoderm of conceptuses. However, one of the above-mentioned *SLC* family members is a different type of transporter, i.e., a sodium dependent glucose transporter (SGLT). The two known members of the SGLT family are *SGLT1* and *SGLT2*, which belong to the *SLC5A* gene family. Similar to the *SLC* and GLUT protein nomenclature, *SLC5A1* or *SLC5A2* are gene names and SGLT1 or SGLT2 represent the corresponding proteins. This type of Na<sup>+</sup>-glucose cotransporter transports glucose against its concentration gradient by coupling its uptake with Na<sup>+</sup> that is simultaneously transported down its concentration gradient. This is the major mechanism for glucose entry into epithelial cells via SGLTs. Glucose can move outward through the basal lateral membrane via GLUT and this action does not require Na<sup>+</sup> (Lee, et al. 2007). Facilitative glucose carriers accelerate the transport of glucose down its concentration gradient by facilitative diffusion, a form of passive transport (Banerjee, et al. 2009, Raja, et al. 2012). cDNAs have been isolated from human tissues encoding a Na<sup>+</sup>-glucose-cotransporter and five functional facilitative glucose transporter isoforms in human tissues have been identified (Bell, et al. 1990). As a tissue responsible for the rapid and substantial transport of nutrients and molecules, the placenta resembles the intestinal

mucosa and renal epithelium, where SGLTs were first discovered as acting via flux coupling (glucose cotransport with Na<sup>+</sup>) (Miller and Bihler 1961, Rajan, et al. 2006, Stulc 1997). Certainly the transport of glucose and the expression of glucose is complex during pregnancy. Materno-placento-fetal transport of glucose may contribute to changes in GLUT1 protein levels, thereby regulating placental and fetal growth (Das, et al. 1998). Hyperglycemia has been reported to increase the renal SGLT activities through the reactive oxygen species-nuclear factor-kappa B pathways (Lee, et al. 2007). Similarly, 72 h of IUGR, ischemic hypoxia, maternal and fetal hypoglycemia led to a 30-50% decline in placental GLUT1 level in ewes (Das, et al. 1998).

### **Amino Acids and Their Transporters**

Amino acids are necessary for all living cells and organisms; and their transport across plasma membrane depends on specialized transporters. Many amino acids have been identified as essential for fetal-placental growth and development including arginine, which is an essential amino acid for conceptus growth and development (Li, et al. 2010, Wu, et al. 2004, Wu and Meininger 2002).

A sufficient supply of arginine to the conceptus is particularly important for ruminants and pigs (Guillomot, et al. 1993). Increasing L-arginine supplement enhanced the reproductive performance of pigs while arginine deficiency is a major factor limiting the growth of milk-fed piglets. Maternal undernutrition and hypercholesterolemia in pigs are associated with reduced concentrations of arginine in the conceptus as well as impaired placental and fetal growth. Polyamines synthesized from ornithine via the

arginase pathway and are also important factors placental growth (Mateo, et al. 2007, Schoknecht, et al. 1994, Wu, et al. 2007, Wu, et al. 1998).

The transfer of amino acids across the syncytiotrophoblast layer of the placenta involves mediated transport mechanisms at the microvillous and basal membrane in addition to diffusion (Smith, et al. 1992). Amino acid transport proteins can be distinguished by substrate specificity. Cationic amino acids such as L-arginine, L-lysine and L-ornithine share the same transporter with a sodium-independent activity, called system y(+) transport (Hosokawa, et al. 1999, McGivan and Pastor-Anglada 1994). The transport of L-arginine by system y(+) generated special interest and focus because of its potential role in the regulation of nitric oxide synthesis in various types of cells (Lopes, et al. 1994, Southam, et al. 1991). Arginine modulates nitric oxide (NO) production and blood flow in the vasculature; and endothelium-derived NO plays an important role in enhancing blood flow and angiogenesis pregnancy, hence increasing the delivery of essential nutrients from the mother to the fetus (Sladek, et al. 1997). Vascular endothelial growth factor (VEGF) stimulates the release of NO from cultured HUVECs and upregulates the expression of NO synthase (NOS) (Hood, et al. 1998, van der Zee, et al. 1997). Endogenous NO produced by nitric oxide synthase (NOS) is partially dependent on the cellular uptake of the NOS substrate L-arginine (L-Arg), which indicates that the cellular L-Arg transport mechanism plays an important to mediate NO-dependent function (Kakoki, et al. 2006). The *SLC7* family members include the cationic amino acid transporters (CATs) and the L-type amino acid transporters (LATs). Both are known to play central roles in human physiology and are involved in diseases such as

viral infection and cancer. Cationic or positively charged amino acids are transported into the cells for protein synthesis as well as conversion of arginine into NO (by NO synthase 2) and polyamines (by arginase) (Fotiadis, et al. 2013). CAT proteins are amongst the first mammalian amino acid transporters identified at the molecular level and seem to be the major entry path for cationic amino acids in most cells. It is known that malfunction of transporter proteins can affect systemic homoeostasis resulting in a number of human diseases. For example, recessive mutations in the *SLC7A7* gene can induce inherited aminoaciduria (Toivonen, et al. 2013).

The expression of the CAT proteins is highly dependent on levels of transcription, mRNA stability, translation and localization (Closs, et al. 2006). In sheep, *SLC7A1* mRNA was most abundant in endometrial luminal (LE) and superficial glandular (sGE) epithelia on Day 16 of the estrous cycle and on Days 16–20 of pregnancy (Gao, et al. 2008a). *SLC7A2* is one of the novel P4 induced and IFNT stimulated genes in sheep, along with previously mentioned facilitated glucose transporter member 1 or *SLC2A1* (Bazer, et al. 2012b). The level and the source of dietary amino acids affect the expression of cationic amino acid transporters in pigs fed wheat-based diets (García-Villalobos, et al. 2012).

### **Summary and Objectives**

Bone marrow–derived circulating endothelial progenitor cells play a key role in blood vessel repair and neovascularization. Because endothelial progenitor cells move towards injuries within blood vessels, detecting their presence can lead to a diagnostic

system for various vascular diseases. Methods for isolating EPCs from the bloodstream have been developed (Plouffe, et al. 2009). Discovery of EPCs has inspired interests in the field of vascular biology and their role in cardiovascular, renal and reproductive physiology and in diseases ranging from rheumatoid arthritis to preeclampsia (Caiado and Dias 2012, Robb, et al. 2007). EPC angiogenic behavior *in vitro* can be determined by expression levels of integrin receptors ITGA5B1, ITGA6B1, ITGAVB3 and ITGAVB5. OPN, which binds these integrins may be involved in angiogenesis by upregulating endothelial cell migration in cooperation with growth factors such as VEGF (Takahashi, et al. 2002), OPN also binds to integrins ITGAVB3 and ITGA5B1 and induces focal adhesion assembly, adhesion and migration of conceptus trophoctoderm cells during implantation (Bazer, et al. 2012a, Caiado and Dias 2012). Together, maternal nutrients including glucose, amino acid (e.g. arginine) and OPN mediate growth, migration, cytoskeletal remodeling and adhesion of trophoctoderm essential for pregnancy recognition signaling and implantation (Bazer, et al. 2012a). In this regard, angiogenesis at the fetal-placental interface plays a critical role in nutrient transport in this regard and circulating EPC incorporation is very likely to be involved. Since conceptuses (embryo/fetus and associated membranes) and uterine endometrium do not carry out gluconeogenesis, transport of glucose from the maternal circulation into the uterine lumen is essential for pregnancy (Schneider, et al. 1981). Unlike sheep, whose GLUTs and SGLTs have been identified in the uterus and peri-Implantation conceptuses, there is limited information about the temporal and spatial expression of these amino acid transporters during pregnancy in pigs. Mechanisms for glucose and amino transport



into the uterine lumen and uptake by conceptuses are not established. *SLC2A1*, *SLC2A4* and *SLC5A1* (mRNA encoding GLUT1, GLUT4 and SGLT1) are present in the LE/sGE and GE of pregnant ewes and *SLC5A1* mRNA levels were higher in ewes treated with P4 (Gao, et al. 2009). GLUTs facilitate the transport of glucose across the plasma membranes of mammalian cells. SGLTs are sodium-dependent glucose transporters that utilize a cotransport mechanism. SGLT1 increases absorption of d-glucose and trigger the glucose-induced secretion cytokines and the upregulation of GLUT2 (Gorboulev, et al. 2012). In addition to glucose, amino acids, such as arginine, are essential for desired fetal growth as well (Li, et al. 2010). Arginase-1 activity is known to increase cell proliferation and also to regulate the production of NO (a major mediator of angiogenesis). CATs are important regulators of NOS and arginase-1 because they regulate L-arginine availability. L-arginine transport is mediated primarily by the Na-independent System  $\gamma(+)$  for cationic amino acids, which has low affinity and high capacity in cells (Gao, et al. 2008a). *SLC7A1* and *SLC7A2* (mRNA encoding CAT1 and CAT2) were identified in mouse blastocysts (Gould, et al. 1991). Little information is available on developmental changes of CATs in uteri or conceptuses of mammals and their affinities for basic amino acids vary greatly (Santos, et al. 2000, Scheepers, et al. 2004).

The specific objectives of this thesis and the accompanying hypotheses are (1) to identify and determine the physiological mechanisms that promote vascular growth in the placenta. The central hypothesis is that OPN binds to EPC integrins, and recruits EPCs to sites of neovascularization during pregnancy. The research plan will focus on

the communication between EPCs and HUVECs that allows EPCs to incorporate into established vascular networks. Previous literature has shown isolated and characterized EPCs from the peripheral blood of newborn pigs and shown their expression of integrins (Erikson, et al. 2009). We hypothesized that EPCs use specific integrins as transmembrane receptors to adhere *in vitro* on OPN and the number of EPCs that invade the matrix in an established 3D collagen matrix system (Bayless, et al. 2009) may increase in a dose-dependent manner in the presence of OPN. (2) to determine the temporal and spatial expression of glucose transporter *SLC5A1* and amino acid transporters *SLC7A1-3* mRNAs in the endometria and placentae of cyclic and pregnant gilts along with the change in levels of glucose, and amino acids within the uterine lumen of cyclic and early pregnant gilts. Successful glucose and amino acid supply via angiogenesis to the growing fetus are known to be important for fetal growth and development. We hypothesize that an increase glucose level in uterine flushing may be accompanied by expressional change of *SLC5A1* in the endometrium, which may be regulated by E2 secreted in during the peri-implantation in pigs. We also hypothesized that the expression profile of *SLC7A1-3* mRNA in the placenta may explain the currently known physiology of an increased arginine transport across the pig placenta in gestational period up to day 60 (Wu, et al. 1995).

CHAPTER II

OSTEOPONTIN BINDS TO THE ITGAV (THE A<sub>v</sub> INTEGRIN SUBUNIT)  
PROMOTING PORCINE ENDOTHELIAL PROGENITOR CELL INCORPORATION  
INTO DEVELOPING VASCULATURE

**Introduction**

Angiogenesis is the formation of new blood vessels from the existing vasculature and is a key step in tissue development, tumor progression and wound healing (Carmeliet 2003, 2004, Folkman and D'Amore 1996). Angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are powerful proangiogenic stimuli, and multiple studies have demonstrated the involvement of these growth factors and their receptors in mediating angiogenic events. Mice lacking VEGF and its receptors fail to form a functional vasculature (Ferrara, et al. 1996, Fong, et al. 1995, Shalaby, et al. 1995). However, several additional key cell signaling pathways have been identified to mediate angiogenesis and are initiated by integrins and other cell surface receptors, growth factors, lipids and the extracellular matrix (ECM). Integrins are heterodimeric transmembrane cell receptors composed of noncovalently linked  $\alpha$  and  $\beta$  chains that serve as a link between the cellular cytoskeleton and ECM (Giancotti and Ruoslahti 1999). The ITGA2B1 ( $\alpha_2\beta_1$ ) integrin is an endothelial receptor for collagen type I (Elices and Hemler 1989) and is necessary for angiogenesis both *in vivo* and *in vitro* (Davis and Camarillo 1996, Drake and Little 1991, Senger, et al. 2002). Other integrins, including ITGA1B1, ITGAVB3 and ITGA5B1, have also been

demonstrated to be involved in angiogenesis (Bayless, et al. 2000, Davis and Bayless 2003, Senger, et al. 1997). Mice lacking *ITGAV* (Bader, et al. 1998) and *ITGA5* (the  $\alpha_5$  integrin subunit) receptors (Yang, et al. 1993 ) result in an embryonic lethal phenotype. Thus, these studies support a key role for integrins in shaping vascular formation in the embryo and adult.

During pregnancy, uterine and placental blood vessels grow and/or dilate to facilitate maximal transfer of nutrients from maternal to placental vasculatures for hematotrophic support of the developing conceptus. OPN is a candidate ECM protein for promoting angiogenesis in the uterus during pregnancy. In gilts, OPN expression increases in scattered cells in the stratum compactum directly beneath the uterine luminal epithelium (LE) between Days 9 and 12, and again between Days 30 and 40 of gestation, where angiogenesis is actively occurring within the pregnant uterus (Garlow, et al. 2002). OPN has also been linked to angiogenesis in other systems. OPN induces tumor cell-induced angiogenesis in mice (Hirama, et al. 2003, Takahashi, et al. 2002), and enhances vascularization in ectopic bone (Asou et al., 2001). However, the precise role of OPN in facilitating angiogenesis during porcine pregnancy remains unknown.

Blood vessels are structurally comprised of a luminal layer of endothelial cells that is surrounded by a basement membrane and vascular smooth muscle cells that form the vessel wall. Recently, senescent sloughed endothelial cells and endothelial progenitor cells (EPCs) have been identified as normal components of circulating blood and may be involved in numerous pathologies (Bertolini, et al. 2007, Bertolini, et al. 2006, Kawamoto and Losordo 2008). EPCs are mononuclear endothelial cell precursors

originating from the hematopoietic compartment of bone marrow that are mobilized into the circulation, where they are thought to be recruited to areas of angiogenesis (Asahara, et al. 1999, Brunt, et al. 2007). Several studies have shown that EPCs are involved in endogenous neovascularization of ischemic tissues (Brunt, et al. 2007, Dimmeler, et al. 2005, Dzau 2005). EPCs from blood can be isolated and identified based on *in vitro* adhesion characteristics and by cell surface markers. As EPCs differentiate in culture, they acquire endothelial cell markers such as VE-CAD, PECAM-1 and von vWF (Parant, et al. 2009, Urbich and Dimmeler 2004). However, no cell surface markers unique to EPCs have been identified that allow definitive isolation of these cells (Hirschi, et al. 2008). Asahara and coworkers identified EPCs as a population of CD34 expressing cells that were more adherent to fibronectin than type I collagen and displayed a spindle-shaped morphology (Asahara 1997). Lin et al. isolated peripheral blood mononuclear cells from patients that had undergone bone marrow transplantation and reported the emergence of highly proliferative endothelial cells 14 to 21 days after isolation from a marrow origin (Lin, et al. 2000). These cells were called endothelial colony forming cells (ECFCs) and displayed characteristics of EPCs, as well as the ability to form blood vessels *in vivo* (Melero-Martin, et al. 2007, Shepherd 2006, Yoder, et al. 2007).

OPN is well-suited to recruit EPCs to sites of active angiogenesis. OPN can regulate various monocyte functions, including the recruitment of proangiogenic monocytes (Leali, et al. 2003), prevention of reverse transmigration (recirculation), and enhancement of survival of cells of a monocyte/macrophage lineage (Burdo, et al. 2007). Supernatants from OPN-treated monocytes are highly angiogenic (Naldini, et al. 2006).

OPN-/- mice exhibit significantly decrease in total hind limb vascular volume after induction of hind limb ischemia compared with wild type mice, suggesting that OPN is needed for collateral vessel formation and successful angiogenesis (Duvall, et al. 2007). In addition, a 32 kDa OPN fragment produced by matrix metalloproteinase (MMP) cleavage binds strongly to the surface of endothelial cells (Gao, et al. 2004). These data imply that OPN modulates the migration and proangiogenic activity of mononuclear and endothelial cells in the progression of new blood vessel formation.

OPN and EPCs are both upregulated during inflammation, pathologic angiogenesis and pregnancy. OPN is chemoattractant to epithelial cells, which is what EPCs differentiate into (Apte, et al. 2005, Asahara 1997, Asou, et al. 2001, Bailey, et al. 2010, Buemi, et al. 2007, Caiado and Dias 2012, Denhardt and Guo 1993, Duvall, et al. 2007, Erikson, et al. 2009, Garlow, et al. 2002, Gomer 2008, Hristov, et al. 2003, Matsubara, et al. 2006, White, et al. 2005). Based on the above characteristics of OPN and EPCs, we hypothesize that OPN expressed within the stratum compactum stroma of the pig uterus during early pregnancy functions to recruit EPCs to sites of neovascularization. The objectives of this study were to isolate and identify EPCs from peripheral pig blood and determine the effect of OPN on EPC adhesion and invasion *in vitro*.

## Materials and Methods

### *Experimental Design*

Experimental and surgical procedures involving animals as a source of porcine endothelial progenitor cells (EPCs) as well as uterine/placental frozen sections complied with the Guide for Care and Use of Agricultural Animals and were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.

### *Study One*

Blood was obtained from newborn piglets, and used to isolate and culture EPCs. RNA was extracted using RNeasy® Mini Kit (Qiagen, Valencia, CA) for RT-PCR analysis to identify *CD31*, *VEGFR2*, *CDH5*, *CD14*, *CD34*, *CD45* and integrins subunits *ITGAV*, *ITGA4*, *ITGA5*, *ITGB1*, and *ITGB5* ( $\alpha_v$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\beta_1$  and  $\beta_5$ ). Endothelial markers vWF, PECAM and VE-CAD were evaluated on the purified cells by immunofluorescence analysis. Affinity chromatography and immunoprecipitation were performed using biotin-labeled EPC extracts mixed with OPN-Sepharose (Sigma, St. Louis, MO) to test whether the ITGAV (the  $\alpha_v$  integrin subunit) on EPCs binds directly to OPN.

### *Study Two*

*ITGAV* (the  $\alpha_v$  integrin subunit) or *GAPDH* expression in EPCs was silenced using siRNA (75 nM) (Ambion, Carlsbad, CA) and knockdown of protein was confirmed by western blot analysis. Immunofluorescence staining against ITGAV and other focal

adhesion proteins including FAK, paxillin and vinculin were also analyzed. Adhesion assays were performed to investigate the ability of EPC's to bind recombinant rat OPN (rOPN) and bovine milk OPN (bOPN).

### *Study Three*

Human umbilical vein endothelial cells (HUVECs) were cultured on a monolayer at the surface of 3D collagen matrices as previously described (Bayless, et al. 2009). In this model, endothelial cells can invade to the matrix and form multicellular tubes with open lumens and branching structures similar to angiogenic vessels *in vivo*. Incorporation of EPCs into invading HUVEC structures were observed in HUVEC-EPC cocultures. We tested whether the addition of soluble OPN enhanced incorporation of EPCs labeled with the fluorophore DiI into invading HUVEC structures.

### *Study Four*

This experiment was designed to evaluate whether OPN binds ITGAV on EPCs to increase EPC incorporation in coculture with HUVEC. HUVEC were cultured with control EPCs or EPCs treated with siRNA directed to *ITGAV* or *GAPDH*. Following gene knockdown EPCs were labeled with DiI and placed in co-cultures with HUVEC in the presence of 0, 30 and 100 µg/ml OPN. Cells were collected for immunofluorescence analysis and protein samples were extracted from all groups to confirm successful knockdown of *ITGAV* or *GAPDH* as described for study two.



### *EPC Isolation and Culture*

The porcine endothelial progenitor cell line pEPC was isolated and cultured following a combination of two methods for purifying EPCs from human blood (Brunt, et al. 2007, Hirschi, et al. 2008). Whole blood (50 c.c.) was obtained from piglets within 6 h of birth, diluted 1:1 in PBS and layered over Ficoll-Paque (Invitrogen, Carlsbad, CA). Following centrifugation (400 x g, 20 min), the mononuclear cell layer was transferred to a new tube and washed twice with sterile phosphate-buffered saline (PBS) (1500 x g, 5 min). Cells were then resuspended in medium-199 (M199) containing 100 µg/ml heparin (Sigma, St. Louis, MO), 0.4 mg/ml lyophilized bovine hypothalamic extract (Pel-Freeze Biologicals, Rogers, AK) and 15% fetal bovine serum (Lonza, no. 14-471F, Walkersville, MD) and cultured on flasks coated with 5-10 µg/ml fibronectin (Invitrogen, Carlsbad, CA) in this medium. After one h, nonadherent cells were removed, the flask was washed, and fibronectin-adherent cells were cultured for 14-21 days or until a confluent monolayer of cells was obtained. At this time, cells were passaged onto gelatin (Sigma-Aldrich, cat. no. G2500, St. Louis, MO)-coated flasks (1 mg/ml) and used for the experiments outlined below.

### *Immunofluorescence*

EPCs were seeded onto four-well chambered slides coated with fibronectin and cultured overnight (37°C, 5% CO<sub>2</sub>). Cells were washed once in PBS and fixed in 2% paraformaldehyde (PFA) (Electron Microscopy Sciences, cat. no. 15712, Hatfield, PA)) in PBS, then permeabilized in PBS containing 1% Triton X-100. Immunofluorescence

staining was performed as previously described (Johnson, et al. 2001). After washing with PBS containing 0.3% vol/vol Tween 20, cells were blocked with 10% vol/vol goat serum and incubated overnight at 4°C with 20 µg/µl rabbit polyclonal antibodies to vWF, VE-CAD (Enzo Life Sciences, Farmingdale, NY) or PECAM (Santa Cruz, Dallas, Texas). Tissue-bound primary antibodies were detected with goat anti-rabbit IgG Alexa 488 (Chemicon, Temecula, CA). Rabbit IgG at the same concentration as the primary antibody was used as a negative control. Slides were overlaid with ProLong<sup>®</sup> Gold antifade reagents with DAPI (Life Technologies –Invitrogen, cat. no. P-36931, Carlsbad, CA) and a cover glass.

#### *Integrin Affinity Chromatography*

To identify porcine EPC (pEPC) integrins that directly bind to OPN, affinity chromatography experiments were performed (Bayless, et al. 1997, Bayless, et al. 1998, Erikson, et al. 2009). Bovine milk OPN extracted as previously described (Bayless, et al. 1997) was coupled to cyanogen bromide-activated Sepharose<sup>®</sup> 4B (Sigma, St. Louis, MO) at 1 mg/ml according to the manufacturer's instructions. Surface biotinylation of pEPCs was performed as previously described (Bayless, et al. 1998, Erikson, et al. 2009). Porcine EPCs were surface-labeled with biotin in 75 cm<sup>2</sup> flasks for 1 h at room temperature and washed with PBS. Cells were lysed with 50 mM octyl-β-D-glucopyranoside (OG) (Fisher Scientific, Pittsburgh, PA) containing 1.5 mM MnCl<sub>2</sub> and MgCl<sub>2</sub> for 30 min on an orbital shaker at 4°C. Cell extracts were centrifuged and then mixed at 20 min intervals with OPN-Sepharose<sup>®</sup> (0.5 ml) for 2 h at 0°C. The column

was washed with 20 ml of 1% OG plus  $Mg^{2+}$  and  $Mn^{2+}$ , and 0.5 ml fractions were eluted with 4 ml of 1% wt/vol OG + 10 mM EDTA. Thirty  $\mu$ l of each fraction were separated on a 7% polyacrylamide gel under non-reducing conditions, transferred to polyvinylidene difluoride (PVDF) and blocked for 30 min with 5% wt/vol nonfat dry milk in Tris-buffered saline containing 0.1% vol/vol Tween 20. Blots were probed for biotin using streptavidin-alkaline phosphatase as previously described (Bayless, et al. 1997, Bayless, et al. 1998).

#### *Integrin Immunoprecipitation*

Protein samples were incubated with 2  $\mu$ g of the rabbit antisera directed to integrins ITGA4 (AB1924), ITGA5 (AB1928), ITGAV (AB1930), ITGB1 (MAB1981;), ITGB3 (AB1968), ITGB6 (the  $\beta_6$  integrin subunit) (MAB2076Z), ITGAVB3 (MAB1976Z), or ITGAVB5 (MAB1961) (All from Chemicon, Temecula, CA) in 500  $\mu$ l of lysis buffer containing 0.5% Nonidet P-40 overnight at 4 °C with gentle shaking. 20  $\mu$ l of protein G Dynabeads® (Invitrogen, cat. no. 10003D, Carlsbad, CA) were added to each sample, incubated for another 2 h, and washed extensively. Magnetic beads were suspended in 1 $\times$  Laemmli sample buffer (Santa Cruz Biotech, cat. no. 161-0737, Dallas, TX) containing 2%  $\beta$ -mercaptoethanol for Western blot analyses.

#### *RT-PCR Analyses*

Partial mRNAs (3  $\mu$ g) for pEPCs were amplified by RT-PCR using tRNA from different passages. *CD14*, *CD31*, *CD34*, *CD45*, *VEGFR2*, *CDH5*, *ITGAV*, *ITGA4*, *ITGA5*,

*ITGB1*, *ITGB3*, *ITGB5*, *ITGA2* ( $\alpha_2$  subunit), *ITGA10* ( $\alpha_{10}$  subunit), *ITGA11* ( $\alpha_{11}$  subunit) primers (Invitrogen, San Diego, CA.) were derived from the swine the swine mRNA coding sequence. All products were amplified along with a beta-actin control using a SuperScript® III Reverse Transcriptase Kit (Invitrogen, cat. no. 18080-051 San Diego, CA). The PCR amplification procedure were : 1) 95°C for 2 min; 2) 58°C for 30 sec, 50°C for 45 sec, and 72°C for 1 min for 25 cycles; and 3) 72°C or 7 min. All samples were loaded in 2% agarose gel containing Bio-Safe (Bio-Rad, Hercules, CA) for electrophoresis at 100 V for 1 h. Images were captured by UVP PhotoDoc-It™ Imaging Systems fitted with a Canon A480 digital camera in benchtop UV transilluminator (UVP, LLC Upland, CA).

The following gene-specific primer sets were used: *CD14* (5'- GTTGCTGCTG CTGCTGCC-3' and 5'-AAGTTGCAGACGCAGCGGA-3'). *CD31* (5'-GAACGGAAG GCTCCCTTGA -3' and 5'-AGGGCAGGTTTCATAAATAAGTGC -3'). *CD34* (5'- GATTGCACTCGTGACCTCGG-3' and 5'-TCCGTGAATAAGGGTCTTCGC-3'). *CD45* (5'-TGGTGGAATACAATCAGTTTGGAG-3' and 5'-CCAATGT GCTGTGTC CTCCAG-3'). *VEGFR2* (5'-TCACA ATTCCAAAAGTGATCGG-3' and 5'-GGTCA CTAACAGAAGCAATAAATGG-3') *CDH5* (5'-CAACGAGGGCATCATCAAGC-3' and 5'-TCGATGGTGGGGTCTGCGG-3'. *ITGAV* (5'-CTGGTCTTCGTTTCAGTGTG C-3' and 5'-GCCTTGCTGAATGAACTTGG-3'). *ITGA4* (5'-CAGATGGGATCTCGT CCACC-3' and 5'-TCTGCTGGACACCTGTATGC-3'). *ITGA5* (5'-GAGCCTGTGGA GTACAAGTCC-3' and 5'-CCTTGCCAGAAATAGCTTCC-3'. *ITGB1* (5'-GACCTGC

CTTGGTGTCTGTGC-3' and 5'-AGCAACCACACCAGCTACAAT-3'). *ITGB3* (5'-AGATTGGAGAACACGGTGAGC-3' and 5'-GTACTTGCCCGTGATCTTGC-3'. *ITGB5* (5'-TCAACAAGTTCAACAAGTCCTACAA-3' and 5'-ATCTCAGCAGTTCA GTGAGAAGAC-3'). *ITGAI1* ( $\alpha_{11}$  subunit) (5'-TGCTCAGCTTCAATGTGTCC -3' and 5'-GCAGCTCCAGCACATTATCA-3'). *ITGA10* ( $\alpha_{10}$  subunit) (5'-TCATTCAGATTCC TTCTACATCCTC -3' and 5'-GAATTGTACAGAATGTGTTGTGG-3'). *ITGA11b* ( $\alpha_{11b}$  subunit) (5'-GAGTTCCTACTACACCACGAATG -3' and 5'-ATCTTTTACAGA CCGGGTACATTG -3').

#### *ITGAV and GAPDH Knockdown Using siRNA*

The following small interfering RNA were obtained from Ambion (Carlsbad, CA): *ITGAV* (5'-CCAACUUCAUUAUAGAUAUU-3' and 5'-AAAUCUAUAAUGAAGUUG G-3'). *GAPDH* (5'-GCCUCAAGAUCaucagCAAtt-3' and 5'-UUGCUGAUGAUCU UGAGG-3'). siRNA stocks (5nmol) were resuspended in 50µl nuclease free H<sub>2</sub>O (100 µM) and stored at -20°C. 3.9 µl of siRNA was mixed with 906.1 µl Opti-MEM® (Life Technologies–Invitrogen, cat. no. 31985-062, Carlsbad, CA) (final concentration = 75 nM). In a separate tube, 15.6 µl small interfering RNA carrier siPORT™ amine (Life Technologies–Invitrogen, cat. no. AM4502, Carlsbad, CA) was mixed with 894.4 µl Opti-MEM®. Mixtures containing siRNA and siPORT amine were combined and incubated for at least 10 min at room temperature. 80% confluent pEPCs ( $0.8 \times 10^6$  cells) were resuspended in antibiotic-free 0.8 ml DMEM and seeded onto 1mg/ml gelatin in PBS-coated T25 flasks. Antibiotic-free DMEM was added to adjust the final volume to

5.2 ml. After 8 h of transfection, cultures were spiked with 5.2 ml growth medium without antibiotics. Endothelial growth medium was prepared by combining 500 ml M199, 0.2 g lyophilized endothelial growth supplement, heparin, and 12.5% fetal bovine serum as previously described (Bayless, et al. 2009). Medium was changed after 24 h and the cells were allowed to grow for another 48 h (37°C, 5% CO<sub>2</sub>) prior to use.

#### *Protein Extraction and Western Blot Analysis*

3.8x 10<sup>5</sup> EPCs (40% of a confluent T25 flask) were trypsinized and centrifuged (350 xg, 3.5 min) and were extracted in 400 µl RIPA lysis buffer with protease inhibitors (Sigma, cat. no. P8340, St. Louis, MO) and 3X protein sample buffer. Proteins in cell extracts (30 µl per lane) were separated on 8% SDS-PAGE gels under non-reducing conditions, and transferred to PVDF membrane for 2 h with methanol on ice. Blots were then blocked with 2% milk on the shaker for 1 h at room temperature, incubated with milk containing polyclonal rabbit ITGAV antibody (EMD Millipore, AB1930, Billerica, MA) at 1:1000 dilution, mouse GAPDH (Abcam, AB9483, Cambridge, MA) at 1:10000 dilution, and mouse alpha tubulin (Enzo Life Sciences, cat. no. IMG-80196, Farmingdale, NY) at 1:10000 dilution overnight at 4°C with gentle mixing and then incubated in corresponding rabbit or mouse HRP-conjugated secondary antibodies (Dako, Carpinteria, CA) at 1:5000 dilution in milk for 1 h at room temperature. Immunoreactive proteins were detected using Immobilon Western Chemiluminescent HRP Substrate (2 ml per blot) (EMD Millipore, lit. no. P36599A Billerica, MA) and

blots were developed then scanned by HP Scanjet G3010 Photo Scanner (Hewlett-Packard, Palo Alto, CA).

### *Cell Attachment Assays*

Cell attachment assays were conducted as previously described (Bayless, et al. 1998, Erikson, et al. 2009). High-binding polystyrene microwells (Corning-Costar, no. 3690, Corning, NY) were coated overnight at 4°C with 50 µl of the following proteins in phosphate buffered saline (PBS) (n=3 replicates/treatment): The thrombin fragment of rat OPN containing intact RGD binding sequence (RGD) or the mutated RAD sequence (RAD) (plasmids were a gifts from Dr. Magnus Hook, Texas A&M Institute of Biosciences & Technology) (McFarland, et al. 1995), bovine OPN (bOPN), bovine fibronectin as positive control (bFN) or bovine serum albumin as negative control (BSA). Proteins were coated at concentrations ranging from 0 - 20µg/ml. After blocking each well in 10 mg/ml BSA in PBS (100 µl), 50,000 pEPC cells were added per well and allowed to attach for 1 h (37°C, 5% CO<sub>2</sub>). In all cell attachment experiments, nonadherent cells were removed by washing in isotonic saline and wells were fixed in 4% formalin in PBS. Plates were stained with 0.1% wt/vol Amido black in 10% acetic acid and 30% methanol for 15 min, rinsed and solubilized with 50 µl 2 N NaOH to obtain an absorbance reading at 595 nm which directly correlated with the number of cells stained in each well (Davis and Camarillo 1993, 1996). To demonstrate that binding of pEPCs to OPN was integrin-dependent, an attachment assay was performed in which levels of cations were varied. Polystyrene microwells (Corning-Costar, Corning,

NY) were coated overnight at 4°C with 20 µg/ml of bOPN, rOPN, collagen I, bFN and blocked for 1 h with 10 mg/ml BSA. Cells were washed in cation-free Puck's Saline A (PSA) (Sigma, St. Louis, MO), and resuspended in PSA containing 100 µg/ml BSA. Cells were allowed to attach for 1 h in either PSA with 2 mM  $\text{Ca}^{2+}$  1 mM  $\text{Mg}^{2+}$  or PSA with no cations (n=3 replicates/treatments)

### *Collagen Gel Invasion Assays*

Collagen type I was prepared from rat tail tendons by non-proteolytic isolation using acetic acid (Sigma, cat. no. A6283, Louis, MO) as previously described (Bayless, et al. 2009, Bornstein 1958 , Rajan, et al. 2006). Collagen matrices were prepared on ice by combining 350 µl of collagen type I (7.1 mg/ml), 39µl 10X M199 (Invitrogen, cat. no. 11825-015, Carlsbad, CA), 2.1 µl 5N NaOH, and 609µl M199 (Gibco–Invitrogen, cat. no. 11150, Carlsbad, CA) to reach a final concentration of 2.5 mg/ml. 80 µl of the mixture was added to each 6.5 mm Transwell® with 3.0 µm pore polyester membrane insert in a 24 well plate (Corning-Costar, lot. 12602010, Corning, NY). Confluent pEPCs (passage 22-26) and HUVECs (passage 3-6) were washed with HEPES buffered saline (150 mM NaCl, 50 mM HEPES (pH 7.4) before trypsinization from T75 flasks. Cells were resuspended at 40,000 (HUVEC) and 30,000 (EPCs) cells per 10 µl in M199 based on counts obtained from Cellometer® Auto 1000 (Nexcelom Bioscience, Lawrence, MA) or Bright-line hemacytometer (VWR, cat. no. 15170-168, Radnor, PA). EPCs were labeled in a 1ml volume with 1 µM CellTracker™ CM-DiI (1 µl in 1 ml ( $3 \times 10^6$ ) cells) (Molecular Probes®, cat. no. C-7001, Eugene, OR) for 15 min at 37 °C.



Cells were washed four times with 1ml of M199 following repeated centrifugations (350 xg, 3.5 min) to remove excessive DiI. 10  $\mu$ l of HUVEC (40,000 cells) and DiI-labeled EPC (30,000 cells) were added into each well insert. 100  $\mu$ l suspension of equilibrated M199 containing Reduced serum II (RSII) (Bayless et al., 2009) (1:250), vascular endothelial growth factor (VEGF) (40 ng/ml, R&D, 293-VE, R&D Systems, Minneapolis, MN), basic fibroblast growth factor (bFGF) (40 ng/ml, 234-FSE, R&D Systems, Minneapolis, MN) and ascorbic acid (50  $\mu$ g/ml) were added on the top of each polymerized collagen matrices. 1 ml of the medium was added into the well under each insert with 1  $\mu$ M sphingosine-1-phosphate (S1P) (Avanti Polar lipids, cat. no. 860492, Alabaster, AL). Both top and the bottom media were supplemented with 12-O-tetradecanoylphorbol-13-acetate (TPA) (50 ng/ml) (Sigma, cat. no. P1585, Louis, MO). In experiments testing the effects of OPN, bOPN or PBS vehicle was added into lower chambers at a final concentration of 0, 30, and 100  $\mu$ g/ml. After 24 h of incubation (37°C, 5% CO<sub>2</sub>), the top medium (100  $\mu$ l) was replaced with fresh medium after washing with HEPES. All inserts were washed with PBS without cations, fixed in 4% PFA overnight and then stained with 1 $\mu$ M DAPI (Molecular Probes®, cat. no. D1306, Eugene, OR) for at least 30 min at room temperature (or 4°C overnight) and stored in double-distilled water for further analysis.

### *Cell Counting*

Filter membranes from each insert were removed by scalpel blade prior to pushing collagen matrices outside of each insert with capped 25½G needles (Becton Dickinson,

Franklin Lakes, NJ). All invading cells were counted using an Olympus CKX41 microscope (Olympus, Center Valley, PA) with an eyepiece equipped with a reticle displaying a 10 x 10 grid (Olympus, Center Valley, PA). Monolayers were identified under the fluorescence DAPI channel initially, and then all DAPI or DiI stained cell nuclei below the surface were considered invading cells as the focus was adjusted up and down routinely to avoid duplicate counting. The center field was selected for quantification in each gel (n=3) and the total number was averaged for each experiment.

#### *Statistical Analyses*

Data were processed by analysis of variance (ANOVA) and presented with standard error (SE). Tests of statistical significance were performed using the appropriate error terms according to the expectation of the mean squares for error.

## Results

### *Purified EPCs Developed an Endothelial-like Phenotype in Culture*

RT-PCR analysis of EPCs isolated from newborn piglets indicated that expression of endothelial markers *CD31*, *VEGFR2*, and *CDH5* increased from passage 1 to passage 16, while expression of monocyte markers, *CD14* and *CD45* decreased with passage. The expression of *CD34* remained constant (Fig.2.1a). Thus, these RT-PCR data suggest cultured pig EPCs develop an endothelial-like phenotype in culture.



Fig. 2.1a. RT-PCR of surface markers in cultured porcine EPCs. RNA extracted from EPCs at different passages (P) was subjected to reverse transcription-PCR using primer sets specific for *CD14*, *CD31*, *CD34*, *CD45*, *CD133*, vascular endothelial growth factor receptor 2 (*VEGFR2*), vascular endothelial cadherin (*VE-CAD*; *CDH5*). Expression of *CD31*, *VEGFR2*, *CDH5*, increased from passage 1 to passage 16, while expression of *CD14*, *CD45* decreased with passage. *CD133* were undetectable while *CD34* remained constant.

Immunofluorescence analysis for vWF, PECAM-1 (CD31), VE-cadherin (VE-CAD; CDH5) in isolated pEPCs was performed to confirm RT-PCR data. Photographs shown in Fig 2.1b indicated cobblestone morphology, consistent with an endothelial cell phenotype. PECAM, and VE-CAD were expressed in a pattern consistent with endothelial cell junctions, and vWF localization appeared to be consistent with Weibel-Palade bodies (Wagner, et al. 1982). No staining was seen with IgG control, as expected. Thus, the purified porcine EPCs express endothelial marker proteins at the expected patterns in culture (Fig. 2.1b).

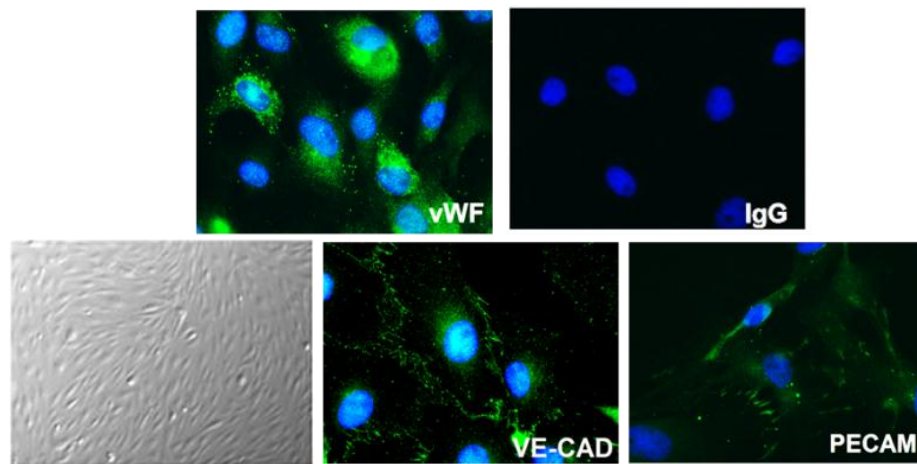


Fig. 2.1b. EPCs express endothelial markers in culture. Differential interference contrast (DIC) image demonstrating morphology of EPCs (width of field = 1000  $\mu\text{m}$ , top left). Immunofluorescence analysis (width of field = 130  $\mu\text{m}$ ) of EPCs shows expression of several endothelial markers including vWF, VE-CAD and PECAM-1. Pre-immune rabbit IgG (rIgG) was used as a negative control.

In addition to testing whether pEPCs expressed endothelial markers, RT-PCR analyses were also conducted to determine integrin expression profiles. Cultured pEPCs (passage 22) express multiple integrin subunits (Fig 2.2a).

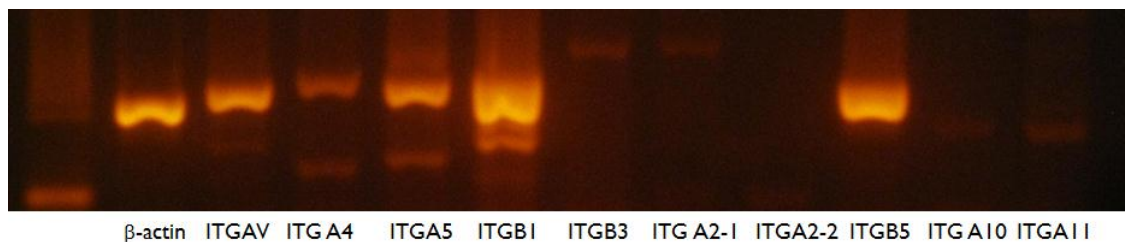


Fig. 2.2a. RT-PCR analysis of integrin subunits expressed in EPCs. RNA extracted from EPCs passage 22 was subjected to reverse transcription-PCR using primer sets specific for the integrin subunits *ITGAV*, *ITGA4*, *ITGA5*, *ITGB1*, *ITGA2*, *ITGA10*, and *ITGA11*. A band indicates the presence of a particular integrin subunit.

To determine which of the various integrin subunits present on EPCs might interact directed with OPN, affinity chromatography was performed using biotin-labeled EPC membrane extracts mixed with OPN-Sepharose beads. Analysis using streptavidin-alkaline phosphatase clearly showed the binding of OPN to integrin  $\alpha$  and  $\beta$  subunit (Fig. 2.2b). Elution fractions E2-E4 were pooled and precipitated using antisera specific to integrin subunits. Immunoprecipitation results indicated that *ITGAV* from EPCs is binding to OPN (Fig. 2.2b).

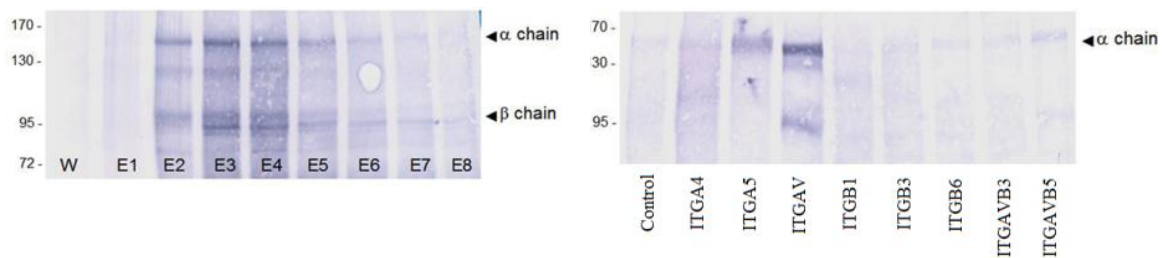


Fig. 2.2b. ITGAV on EPCs directly bound OPN. Left panel: Affinity chromatography experiments. Biotin-labeled EPC membrane extracts were mixed with OPN-Sepharose and bound integrins eluted with EDTA. Right panel: Immunoprecipitation studies. EDTA elution fractions E2-E4 were pooled and combined with various antibodies directed to the integrin subunits indicated and protein G Sepharose overnight. Beads were washed and boiled. Supernatants were separated by non-reducing SDS-PAGE, transferred to PVDF and analyzed using streptavidin-alkaline phosphatase.

#### *ITGAV Silencing Reduced EPC Binding to OPN and Focal Adhesion Assembly*

ITGAV expression in EPCs was confirmed using immunofluorescence (Fig. 2.3a). The expected punctate pattern consistent with localization to focal adhesions was observed.

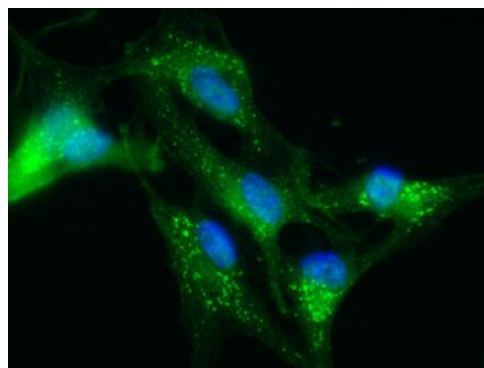


Fig. 2.3a Immunofluorescence staining of ITGAV in EPC. EPCs were stained (width of field = 130  $\mu$ m) using antisera directed to the ITGAV subunit (AB1930; Chemicon, Temecula, CA).

In addition, EPCs attach to recombinant rat OPN in an RGD-dependent manner (Fig. 2.3b), which supports a role for the  $\alpha_v$  integrin, as well as other RGD-binding integrins in recognizing OPN.

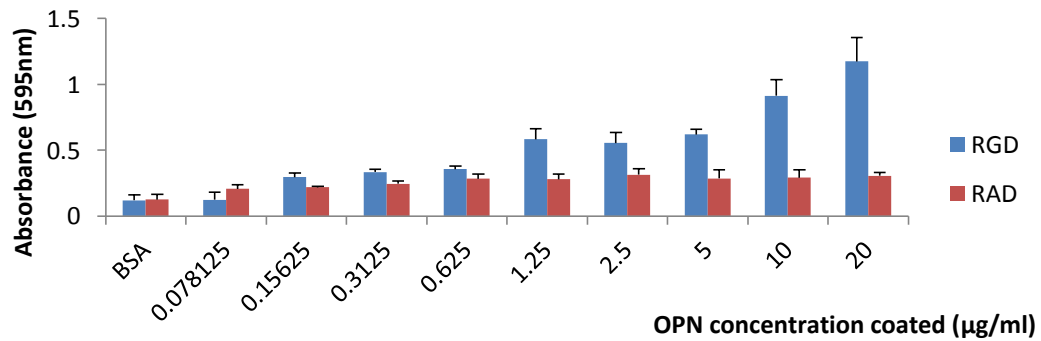


Fig. 2.3b. EPCs dose-dependently bound to recombinant rat RGD in an RGD-dependent manner. EPCs dose-dependently binding to recombinant rat OPN containing an RGD sequence, but not the mutated RAD control, indicating a dependence on the RGD sequence to promote EPC attachment to OPN. Adhesion assays were conducted with recombinant rat OPN with an intact integrin binding sequence (RGD) rat OPN with a mutated integrin binding sequence (RAD). Plates were coated with the indicated concentration of proteins overnight and blocked with BSA. pEPCs were allowed to attach for 1 h before nonadherent cells were removed. Adherent cells in all attachment experiments were fixed, stained with Amido black, and quantified. Values represent absorbance readings (595 nm; 3 wells/data point).

To confirm a functional role for *ITGAV* in mediating EPC attachment to OPN, siRNA experiments were conducted. Decreased expression of *ITGAV* was confirmed in the *ITGAV*-siRNA group using western blot analyses and immunofluorescence staining compared with a *GAPDH*-siRNA control (Fig. 2.4a).

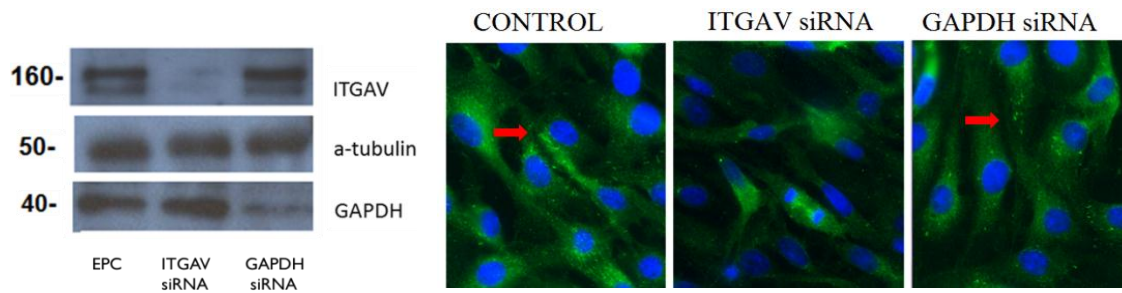


Fig 2.4a. Successful silencing of ITGAV expression in pEPCs using siRNA. Left panel: Western blots confirming successful knockdown of *ITGAV* and *GAPDH* by their respective siRNA targeting sequences (75 nM, 48 h transfection). Proteins in cell extracts (30  $\mu$ l per lane) were separated on 8% SDS-PAGE gels under non-reducing conditions, and transferred to PVDF membrane for 2 h with methanol on ice. Blots were then blocked with 2% milk on the shaker for 1 h at room temperature, incubated with milk containing polyclonal rabbit ITGAV antibody at 1:1000 dilution, mouse GAPDH at 1:10000 dilution, and mouse alpha tubulin at 1:10000 dilution overnight at 4°C and then incubated in corresponding HRP-conjugated secondary antibodies at 1:5000 dilution in milk for 1 h at room temperature. Immunoreactive proteins were detected using Immobilon Western Chemiluminescent HRP Substrate (2 ml per blot) and blots were developed and scanned for analysis. Right panel: Immunofluorescence staining showing *ITGAV* knockdown in EPC. Immunofluorescence analysis confirmed the decreased expression of ITGAV using siRNA directed to *ITGAV*, but not *GAPDH* siRNA controls. The arrows represent the signals of focal adhesion formed using ITGAV (width of field = 130  $\mu$ m).

Fig 2.3b demonstrates pEPCs attach to OPN in an RGD-dependent manner. To test whether the ITGAV is required for binding to OPN, EPCs treated with no siRNA (CON) or siRNA directed to *ITGAV* or *GAPDH* control were tested for the ability to attach to various extracellular matrix proteins. Wells were coated with bOPN, rOPN, bovine fibronectin (bFN), and collagen type I (COL I). Experiments were conducted as described in Fig 1a. Silencing *ITGAV* decreased EPC bind to bOPN, rOPN and bFN (Fig 2.4b). No effect of *ITGAV* siRNA was seen on EPC adhesion to COL I, as ITGAV



integrins are not involved in collagen binding (Fig 2.4a). Consistent adhesion to all substrates was observed with siRNA directed to *GAPDH*, as well as non-transfected CON. Little adhesion was seen on BSA substrate, as expected.

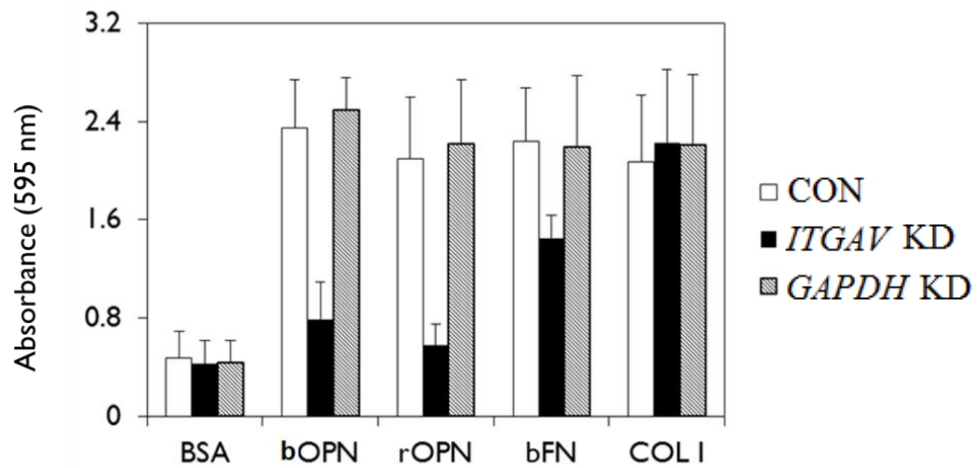


Fig. 2.4b. *ITGAV* is required for EPC attachment to OPN. Adhesion testing whether EPC binding to OPN is *ITGAV* dependent. Adhesion assays were conducted with multiple matrix proteins, including bovine OPN (bOPN), recombinant rat OPN with an intact integrin binding sequence (rOPN), bovine fibronectin (bFN), and collagen type I (COL I). Plates were coated with 20  $\mu$ g/ml of proteins overnight and blocked with BSA. EPCs were allowed to attach for 1 h before nonadherent cells were removed. Adherent cells were fixed, stained with Amido black, and quantified. Bovine serum albumin (BSA) served as a negative control and collagen type 1 (COL1) served as a positive control.) Values represent absorbance readings (595 nm; 3x3 wells/data point).

The cell adhesion experiments in Fig 2.4b indicate a decreased ability of EPCs to attach to bOPN, rOPN, and bFN. Because focal adhesions have been implicated in mediating cell attachment (Dumbauld, et al. 2013), immunofluorescence staining was

conducted with control (non-transfected) EPCs and EPCs treated with siRNA directed to *ITGAV* and *GAPDH* (Fig 2.4c). Following siRNA treatment, EPCs were placed on coverslips and coated with 1mg/ml gelatin and stained for the focal adhesion proteins focal adhesion kinase (FAK), vinculin, and paxillin. Similar staining patterns were observed in CONTROL and *GAPDH* siRNA-treated EPCs, which is consistent with punctate staining patterns of cells in culture. However, EPCs treated with *ITGAV* siRNA showed a decrease in vinculin and paxillin staining, while FAK was not overtly affected. No staining was seen in IgG controls.

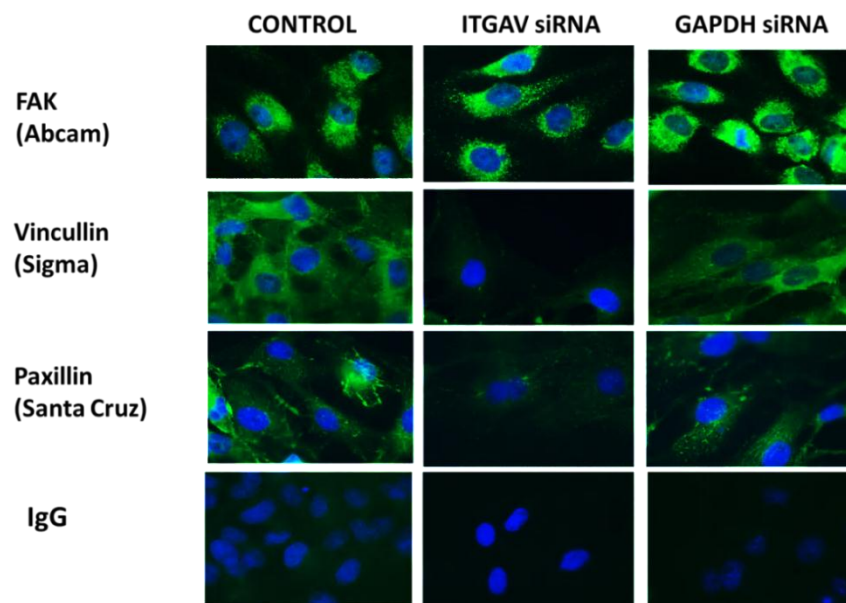


Fig. 2.4c. Silencing of *ITGAV* reduces vinculin and paxillin incorporation into focal adhesions. Immunofluorescence staining of focal adhesion proteins in control (non-treated) EPCs, and EPCs treated with *ITGAV* and *GAPDH* siRNA (75 nM, 48 h transfection). Cells were plated on coverslips and stained with antibodies directed to FAK, vinculin, and paxillin, as well as IgG controls (width of field = 130  $\mu$ m).

*EPCs do Not Invade on Their Own, but Incorporate into Sprouting HUVEC Networks*

EPCs have been reported to immobilize from a bone marrow compartment and respond to angiogenic cues to incorporate into newly-forming angiogenic networks (Asahara, et al. 1999). To test whether EPCs have the ability to assemble into networks on their own, pEPCs, Human umbilical vein endothelial cells (HUVECs), and porcine trophoctoderm (pTr2) cells were placed in invasion assays (Bayless, et al. 2009). HUVECs, EPCs, and pTr2 cells were labeled with DiI and placed on the surface of 3D collagen matrices in the presence of VEGF, bFGF, and S1P for 24 h (Fig 2.5a). Cultures were photographed from the side, and HUVEC were able to invade (arrows) below the original monolayer (\*). EPCs and pTr2 cells, however, had no ability to invade on their own under these conditions. Note only a single monolayer has formed with no invading structures underneath when EPCs and pTr2 cells are cultured alone. The results were similar with HUVEC, EPCs, and pTr cells that were not labeled with DiI (data not shown).

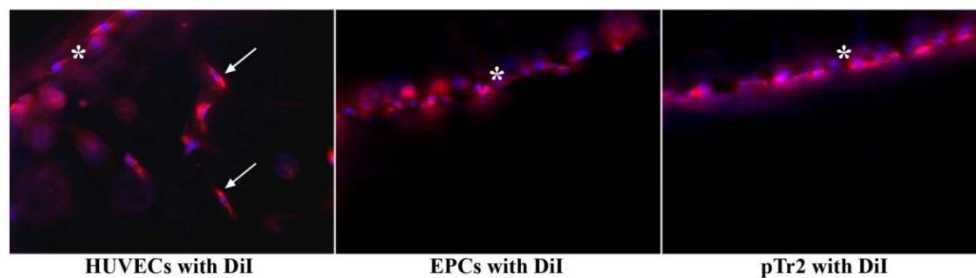


Fig. 2.5a. EPCs have no ability to invade collagen matrices. DiI-labeled HUVECs, EPCs or porcine trophoctoderm (pTr) cells were seeded as confluent monolayers on the surface of collagen gels. Invasion was allowed to proceed for 48 h at which time the gels were fixed, stained with DAPI, cut, mounted, and photographed from the side. Arrows indicate cells invading beneath the monolayer (\*) (width of field = 500  $\mu$ m).

Because EPC incorporation into angiogenic networks *in vivo* requires adult endothelial cells, we next tested whether EPCs can incorporate into sprouting networks. To perform these experiments, EPCs (or control pTr2 cells) were placed in sprouting assays with HUVEC, which readily form sprouts in the presence of pro-angiogenic factors (Fig 2.5a). Unlabeled HUVEC were combined with either EPCs or pTr2 cells labeled with DiI so that invasion of both cell types could be monitored (Fig 2.5b). While EPCs were able to incorporate into sprouting HUVEC networks (see arrows), pTr2 cells had no ability to incorporate and remained on the surface of the collagen matrix as a monolayer (\*). The results from Figures 2.5a and 2.5b are quantified in Figure 2.6.

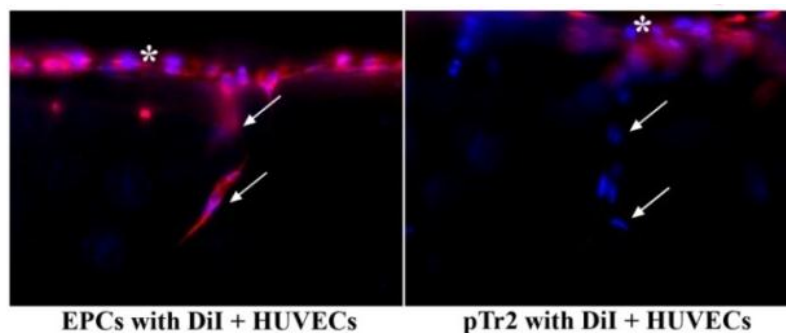


Fig. 2.5b. EPCs, but not pTr2 cells, can incorporate successfully into sprouting endothelial networks. Unlabeled HUVECs (40,000) were combined with DiI-labeled EPCs or porcine trophectoderm (pTr) cells were placed on the surface of collagen matrices in the presence of S1P, VEGF, and bFGF for 48 h. Invasion was allowed to proceed for 48 h at which time the gels were fixed, stained with DAPI, cut, mounted, and photographed from the side. Arrows indicate cells invading beneath the monolayer (\*). Note that labeled pTr cells did not invade when seeded onto gels along with HUVECs, indicating a selective ability of EPCs to incorporate into HUVEC networks (width of field = 500  $\mu$ m).

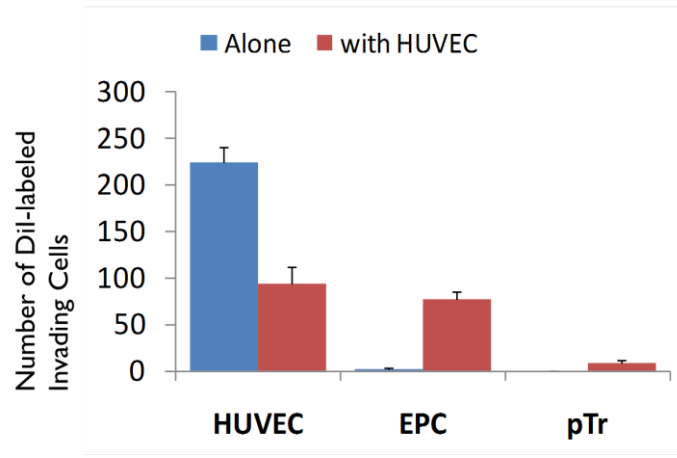


Fig. 2.6. Quantification of EPC invasion into collagen matrices in the presence and absence of HUVEC. The number of DiI labeled invading cells was quantified from experiments shown in Figs 2.5a and 2.5b. Four fields from each treatment group were used to obtain average number of invading cells per  $1\text{mm}^2$  field ( $\pm$  SD). Data shown are representative of  $n=4$  experiments.

#### *EPC Incorporation into HUVEC Networks is Enhanced by OPN*

The assay system described above (Figs 2.4-2.6) allows us to test the original hypothesis that OPN attracts EPCs into sites of active angiogenesis. To perform these studies, invasion experiments were established in the presence of pro-angiogenic growth factors. HUVEC were placed on the surface of collagen matrices in the presence of DiI labeled EPCs to monitor EPC incorporation into sprouting HUVEC networks. In these experiments, OPN was added separately to only invading HUVEC, or to HUVECs and DiI labeled EPCs in co-culture experiments. While OPN did not affect HUVEC

sprouting responses (Fig 2.7, left panel), OPN dose-dependently stimulated EPC incorporation into HUVEC networks (Fig 2.7, right panel).

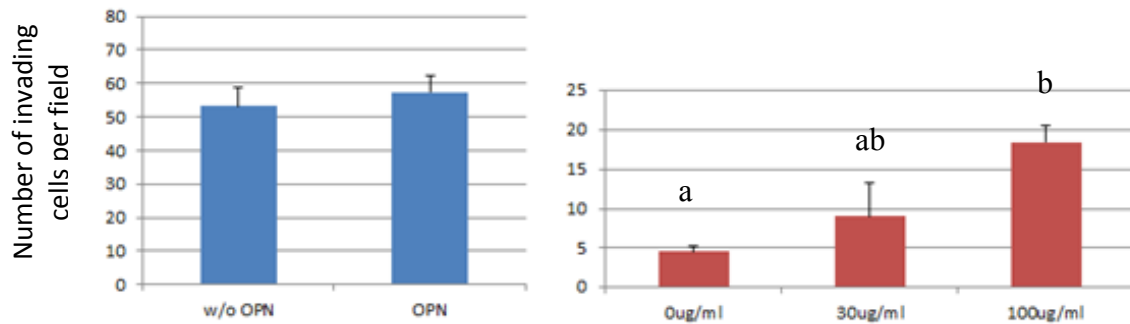


Fig. 2.7. OPN stimulated increased incorporation of EPCs into sprouting HUVEC networks but did not enhance HUVEC invasion. Invasion (48 hr) was stimulated by FGF, VEGF, and S1P in all experiments. Left panel: HUVECs were allowed to invade in the presence of 0  $\mu\text{g/ml}$  (w/o OPN) or 100 $\mu\text{g/ml}$  OPN. Right panel: HUVEC were seeded along with DiI labeled EPCs onto collagen gels, and soluble OPN was added to culture medium in the bottom chamber at the concentrations indicated. Cultures were fixed, stained with DAPI, and the number of invading HUVEC (left panel) and EPCs (right panel) was quantified. Four fields from each treatment group were used to obtain average number of invading cells per 1  $\text{mm}^2$  field ( $\pm$  SD). Data shown are representative of 4 independent experiments (a and b has statistically significant difference, a/b and ab does not).

#### *OPN-stimulated EPC Incorporation Into HUVEC Networks Requires $\alpha_v$ Integrins*

The data in Fig. 2.7 indicate that EPCs respond to OPN. Because ITGAV expressed on EPCs binds OPN directly (Fig. 2.2b) and ITGAV is required for EPCs to attach to OPN (Fig. 2.4b), this raised the question of whether silencing *ITGAV* in EPCs would interfere with OPN-stimulated EPC incorporation into sprouting HUVEC networks. To answer this question, HUVECs were co-cultured in invasion assays with

control EPCs (no siRNA treatment), or EPCs treated with siRNA directed to *ITGAV* (ITGAV-KD) or *GAPDH* (GAPDH-KD). In the absence of OPN, the total number of invading cells (HUVEC and DiI-labeled EPCs) did not change (Fig. 2.8, left panel). EPCs lacking *ITGAV* and *GAPDH* expression also invaded similarly to Control cells (Fig. 2.8, right panel), indicating no role for ITGAV in stimulating EPC incorporation into HUVEC networks in the absence of OPN.

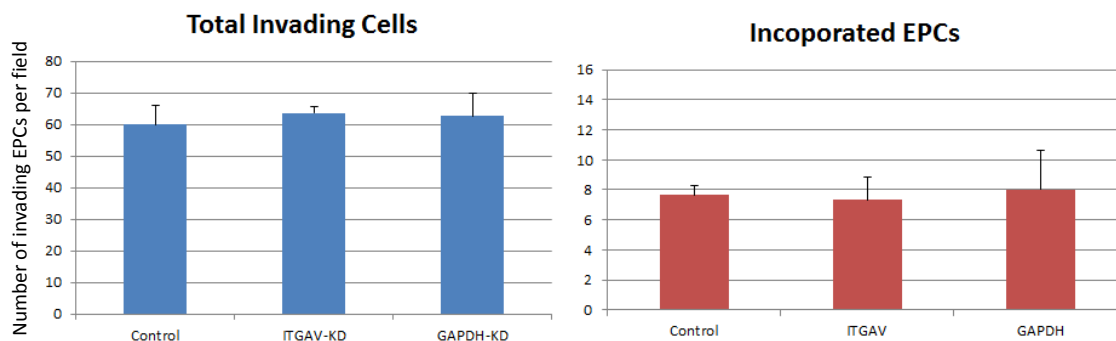


Fig. 2.8. HUVEC and EPC invasion did not change in the absence of OPN. DiI-labeled EPCs expressing no siRNA (Control) or siRNA directed to *ITGAV* (ITGAV-KD) or *GAPDH* (GAPDH-KD) were mixed with unlabeled HUVECs and seeded onto the monolayer of collagen gels containing FGF, VEGF and S1P in the absence of OPN for 48 h before the number of invading cells was quantified. Left panel: Total cell (HUVEC and EPC) invasion in the absence OPN. Right panel: EPC invasion in the absence OPN. Four fields from each treatment group were used to obtain average number of invading cells per 1mm<sup>2</sup> field (+/- SD). Data shown represent 4 independent experiments (n=9, p=0.09581>0.05).

The data in Fig. 2.8 suggest that OPN engages ITGAV on EPCs to stimulate EPC incorporation into sprouting HUVEC networks. To prove this, experiments were

conducted as in Figure 2.8 in the presence of OPN. Control EPCs (no siRNA treatment), or EPCs treated with siRNA directed to *ITGAV* (ITGAV-KD) or *GAPDH* (GAPDH-KD) were tested in invasion assays with HUVEC in the presence of 100  $\mu$ g/ml OPN. In the presence of OPN, the total number of invading cells (HUVEC and DiI-labeled EPCs) did not change (Fig. 2.9, left panel). While EPCs lacking *GAPDH* expression invaded similarly to control cells (Fig. 2.9, right panel), EPCs expressing *ITGAV* siRNA had a decreased ability to incorporate into sprouting HUVEC networks, indicating a requirement for *ITGAV* when EPC incorporation into HUVEC networks was stimulated by OPN. Altogether these data suggest that OPN enhanced EPC incorporation into sprouting HUVEC networks in a manner that required engagement of *ITGAV*.

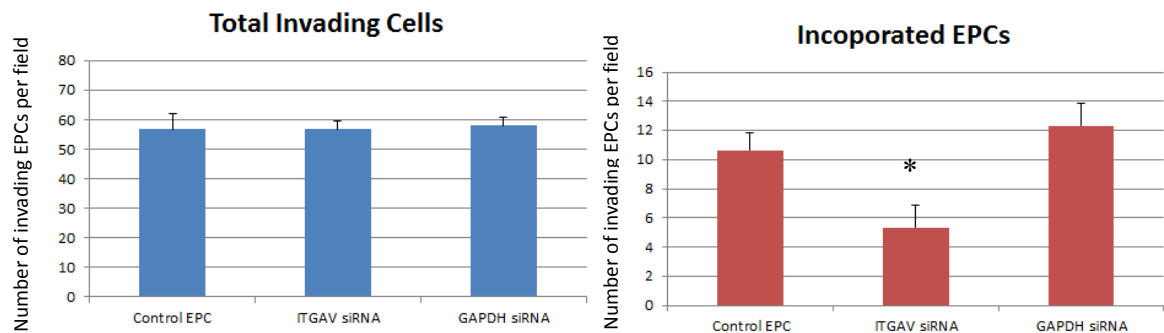


Fig. 2.9. EPC incorporation into sprouting HUVEC network was enhanced by OPN, but HUVEC invasion alone was unaffected. Experiments were conducted as in Fig. 2.8 in the presence of 100 $\mu$ g/ml OPN. Di-I labeled EPCs were seeded with HUVECs onto collagen gels; soluble OPN was added to culture medium in the bottom chamber at the concentrations indicated. Invasion was allowed to proceed for 48 h before the gels were fixed with PFA, stained with DAPI, mounted onto slides and the number of invading EPCs was quantified (\* =  $P < 0.05$  or statistically significant difference).



## **Discussion**

During pregnancy, survival and growth of the fetus depends on nutrients that are transferred from the maternal to fetal circulation through the placenta. Successful pregnancy requires expansion and juxtaposition of the placental and uterine vasculature to facilitate exchange of nutrients, gasses and metabolic wastes between mother and fetus. Angiogenesis, growth of new blood vessels from the existing vasculature, is fundamental to this process. Poor transport of nutrients during development can lead to long-term problems throughout adulthood (Findlay 1986, Jaffe 2000, Zygmunt, et al. 2003). Defective placental and uterine angiogenesis contribute to infertility in several gestational pathologies including gestational diabetes, intrauterine growth restriction, and preeclampsia, as well as increased life-long cardiovascular risk (Barker 2004, Lain and Roberts 2002, Mayhew, et al. 2004, Smith, et al. 2003). In pig placenta, the attachment of fetal and maternal epithelium is associated with distinctive changes in endometrial epithelial cells, and is strengthened by intertwining of villi from chorionic and endometrial epithelial cells and interlocking folds between the two epithelia. Thus, nutrient exchange between mother and fetus must penetrate two epithelial layers, and successful nutrient transfer is highly dependent on new blood vessel formation. As a result, the maternal/fetal interface is a site of rapid and massive blood vessel formation, where robust angiogenic blood vessel formation occurs at the interface (Ford, et al. 1982, Wu, et al. 2010, Wu, et al. 1995, Zygmunt, et al. 2003).

Recruitment of EPCs, once known as angioblasts (Kovacic JC 2008), from bone marrow to peripheral blood occurs in adults (Zygmunt, et al. 2003). EPCs are an

emerging circulating cell type that have been widely studied in cardiovascular medicine for their therapeutic potential. However, little is known about EPC numbers and functions in either normal or abnormal pregnancies in domestic animals. In humans, studies suggested that EPCs may play an important role in the regulation and maintenance of the placenta, maintaining vascular integrity during pregnancy (Buemi, et al. 2007, Savvidou, et al. 2008, Sugawara, et al. 2005a, Sugawara, et al. 2005b). In this study, we find that OPN may aid recruitment of circulating EPC to a developing vascular network. Our findings support the upregulation of OPN in areas actively undergoing angiogenesis during pig pregnancy since OPN transcript was reported in scattered cells of the stratum compactum immediately beneath LE on Day 9 of pregnancy in pigs (Garlow, et al. 2002). These studies provide support for the ability of osteopontin to increase the incorporation of pEPCs into growing vascular networks. The approach utilized a novel *in vitro* model of endothelial cell invasion characterized by assembly of multicellular networks that develop lumens and form angiogenic sprouts (Bayless, et al. 2009). The pig was found to be a useful source of EPCs, which were isolated in adequate numbers from neonates to allow for phenotypic characterization and functional testing in sprouting assays, as well as manipulation of gene expression.

We have isolated and characterized EPCs from the peripheral blood of newborn pigs using a hybrid of previously described methods (Asahara 1997). These EPCs express integrins, and use these transmembrane receptors to adhere and migrate *in vitro* on osteopontin. Our data indicate the ITGAV subunit expressed on EPCs binds to OPN (Fig. 2.3b). *ITGAV* encodes for the ITGAV subunit (CD51 antigen) of the integrin

family, which later forms the vitronectin receptor has a central role in angiogenesis (Rüegg and Mariotti 2003). When the function of *ITGAV* gene is silenced by siRNA, *ITGAV* positive focal adhesions are decreased, as well as other focal adhesion proteins including vinculin and paxillin. Vinculin has been implicated in force transmission mechanosensing (Dufour, et al. 2013). Paxillin is a signal transduction adaptor protein found at the interface between the plasma membrane and the actin cytoskeleton (Turner 2000). Both vinculin and paxillin regulate force transmission and cell-cell or cell-ECM interaction. In this study, EPC binding to bOPN and recombinant RGD decreased with *ITGAV* knockdown, as did the formation of vinculin- and paxillin-positive focal adhesions, suggesting the importance of *ITGAV* in EPC focal adhesion formation and interaction with OPN. *ITGAV*-dependent EPC binding to OPN required the RGD domain, as EPCs did not attach to a mutated RAD (Fig. 2.3a. OPN dose-dependently increase EPC incorporation in actively invading HUVEC in the three-dimensional matrix model, but when *ITGAV*-silenced EPC were cultured with actively invading HUVEC, they fail to invade due to the lack of proper *ITGAV* to bind to OPN. Collectively, these data indicate that OPN promotes attachment and invasion of EPCs through *ITGAV*, enhancing angiogenic responses.

In conclusion, we offer a novel theory of how placental vascularization may be maximized during pregnancy. This includes recruitment of EPCs from the circulation by OPN to incorporate into growing vessels. The exact mechanism of the EPC-endothelium crosstalk is unknown. Silencing of *ITGAV* does not completely block EPC incorporation into sprouting HUVEC networks, suggesting other mechanisms are involved. Previous

studies in our lab showed that soluble factors secreted by HUVEC do not stimulate EPC invasion (data not shown), strongly suggesting that EPC incorporation into vasculature is the result of direct physical communication between EPCs and HUVECs. Since direct contact between cell types is required, it is tempting to speculate that junctional communication between HUVECs and EPCs may play a role in the crosstalk between the two cell types. Adherens, gap, and tight junctions have been shown to play a critical role in angiogenic responses (Gärtner, et al. 2012, Wallez and Huber 2008, Wallez, et al. 2006, Yu, et al. 2011). Thus, one or more of these cell-cell signals may be an important component of HUVEC directed EPC incorporation into new vascular networks. Further work will be necessary to test whether these pathways are involved in directing EPC incorporation into sprouting endothelial networks. Our current incomplete understanding of placental and uterine angiogenesis, and the role of EPCs in this process, is a formidable barrier to development of new therapies to alleviate or prevent prenatal defects. The functional utility of EPCs relies on the availability of an adequate number of EPCs with robust ability to home, migrate, and proliferate in target tissues. EPCs could become a powerful therapeutic tool because of their mobility and the ability to inject intravenously. We hope this study can contribute an understanding of the mechanism through which EPC recruitment occurs, and aid the development of novel strategies to target diseases in pregnancy associated with dysregulated angiogenesis.

CHAPTER III

EXPRESSION AND REGULATION OF GENES FOR GLUCOSE AND ARGININE  
TRANSPORTERS IN PIG UTERI, CONCEPTUSES AND PLACENTAE  
INCREASES DURING PREGNANCY

**Introduction**

Glucose is delivered across cell membranes by either facilitated glucose transport (GLUT family uniporters) or sodium-dependent glucose transport (SGLT family cotransporters) mechanisms. The two most studied transporter proteins in the SGLT family are SGLT1 and SGLT2, which are members of the solute carrier 5A (*SLC5A*) gene family. SGLT1 activity was first discovered in rabbit intestine but expression was low (Hediger, et al. 1987). Recent studies have identified glucose transporter *SGLT1* mRNA expression in epithelial cells on the luminal surface of the large intestine, small intestine, and stomach in mice (Yoshikawa, et al. 2011). These transporters likely provide significant uptake of glucose in the gastrointestinal tract. SGLTs transport glucose across cell membranes using a secondary active transport mechanism that utilizes a sodium-potassium ATPase pump to deliver glucose and galactose against its concentration gradient. This form of glucose transport takes place not only across the luminal epithelium, or apical surface of absorptive enterocytes but also in renal proximal tubules (Wood and Trayhurn 2007).

Amino acids are also delivered across cellular membranes using transporter proteins. The cationic amino acid transporter proteins are a subfamily of the solute

carrier family 7 (*SLC7*). Four transporter proteins for cationic amino acids have been confirmed, including *SLC7A1*, *SLC7A2A*, *SLC7A2B*, and *SLC7A3*, which can be potentially regulated at the transcriptional, mRNA stability, translational and subcellular localization levels (Closs, et al. 2007). Cationic or positively charged amino acids are transported into the cells for protein synthesis as well as conversion of arginine into NO (by NO synthase 2) and polyamines (by arginase) (Fotiadis, et al. 2013). CAT proteins were amongst the first mammalian amino acid transporters identified at the molecular level and are a major entry path for cationic amino acids in most cells. It is known that malfunction of these transporter proteins can affect systemic homeostasis resulting in a number of human diseases.

The functional significance of sodium-dependent glucose and amino acid transporters in the endometrium of the uterus is only beginning to be established. In mammals, secretions of the uterine epithelia, called histotroph, are associated with conceptus survival and development during the crucial peri-implantation period of pregnancy when the conceptus (embryo and associated placental membranes) elongates and remains free-floating in the uterine lumen (Bazer 1975). Reports from sheep suggest roles for nutrients in conceptus growth and development. Glucose and select amino acids, including arginine, leucine, glutamine and glutathione, increase preferentially in the uterine luminal fluids of pregnant ewes as compared to the estrous cycle, and there is dynamic temporal and spatial expression of *SLC7A1*, *SLC7A2* and *SLC5A1* mRNA in ovine uterus and placenta during pregnancy (Gao, et al. 2008b). These transporters are present in uterine LE and GE, their expression is stimulated by P4 and the expression of

*SLC7A1* and *SLC7A2* is further enhanced by IFNT during early gestation. Importantly *SLC5A1* is also expressed in the trophoctoderm and endoderm of sheep conceptuses (Gao, et al. 2008a, Gao, et al. 2009, Gao, et al. 2008b).

Recently we detected increased glucose and select amino acids in the uterine luminal fluids of early pregnant pigs. Further it has been known for some time that there is dramatically increased transport of arginine across the placenta in pigs. We hypothesized that *SLC5A1* expression increases in the endometrium during the peri-implantation period in pigs and serves as a conduit for the transport of glucose into the uterine lumen as a nutrient source for the elongating and implanting conceptus. We also hypothesized that the increased transport of amino acids, including arginine, across the uterine and placental epithelia of pigs is mediated through increased expression of cationic amino acid transporters *SLC7A1-3*. Therefore, we examined the temporal and spatial expression of *SLC5A1*, *SLC7A1*, *SLC7A2* and *SLC7A3* at the uterine/placental interface of pigs as a possible mechanism for increased transport of glucose and arginine from mother to fetus during pregnancy in pigs.

## **Materials and Methods**

### *Experimental Design*

All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

### *Study One*

This experiment measured the change in levels of arginine, leucine and glutamine within the uterine lumen of cyclic and early pregnant gilts. Gilts were randomly assigned to either cyclic or pregnant status. Those in the pregnant group were bred when detected in estrus and 12 h later. Gilts were ovariectomized on either days 5, 9, 12 or 15 of the estrous cycle or Days 9, 10, 12, 13, 14 or 15 of pregnancy (n=4 gilts per Day per status). Uterine horns were flushed with 20 ml of 10 mM Tris buffer (pH 7.0). Pregnancy was confirmed by the presence of morphologically normal conceptuses. Uterine flushings were purified by centrifugation at 3000 xg for 15 min at 4°C, and the supernatants were aliquoted and stored at -80°C until analysis.

### *Study Two*

This experiment determined the temporal and spatial expression of *SLC5A1* and *SLC7A1-3* mRNAs in the endometria and conceptuses of cyclic and pregnant gilts. Gilts were detected in estrus and assigned randomly for ovariectomy on either Days 5, 9, 12 or 15 of the estrous cycle or Days 9, 12, 13, 14, 15, 20, 25, 30, 35, 40, 60 or 85 of



pregnancy (n=3-4 gilts per Day per status). Conceptuses were collected from Days 12, 13, 14, and 15 of pregnancy, subjected to fresh 4% paraformaldehyde in PBS (pH 7.2) fixation, and embedded in Paraplast-Plus (Oxford Laboratory, ST. Louis, MO). Several sections (~0.5 cm) from the middle of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Laboratory, St. Louis, MO). The remaining endometrium was physically dissected from the myometrium, frozen in liquid nitrogen, and stored at -80°C for RNA extraction.

#### *Study Three*

This experiment determined the effects of short-term treatment with estrogen on abundance of *SLC5A1* mRNA in the endometria of ovariectomized gilts. Gilts were detected in estrus and assigned randomly to receive intramuscular injections daily of estradiol benzoate (Sigma, St. Louis, MO) in corn oil (CO) or CO alone (n = 4 gilts per treatment) on Days 11, 12, 13, and 14 of the estrous cycle. Gilts were ovariectomized on Day 15 of pseudopregnancy, and endometrial tissues collected and handled as described in Study 2.

#### *Study Four*

Porcine luminal epithelial (LE) cells were isolated and cultured as previously described (Ka, et al. 2007). Passage six LE cells were cultured in DMEM in T25 flasks until reaching 60% confluency, and then were treated with  $\beta$ -estradiol 3-benzoate (Sigma cat. no. E8515, St. Louis, MO) at 0, 0.05, 0.5, 5, or 50 ng/ml for 24 and 48 h (n =

3 flasks per treatment) under an atmosphere of 45% nitrogen, 5% carbon dioxide, and 50% oxygen.

#### *Analyses of Glucose and Amino Acids in Swine Uterine Flushings*

Uterine flushings (0.5 ml) were deproteinized with an equal volume of 1.5M HClO<sub>4</sub> followed by addition of 0.25 ml of 2 M K<sub>2</sub>CO<sub>3</sub>, because they are the preferred acid and base, respectively, for processing biological samples for analyses of amino acids (Wu and Meininger 2008) and the processed samples also can be analyzed accurately for total glutathione (Wang, et al. 2008). Amino acids in the extract were measured by fluorometric HPLC methods involving precolumn derivatization with o-phthalaldehyde as described previously (Wu, et al. 1997). Total arginine, leucine, glutamine were measured as described for cysteine (Wu, et al. 1997).

The HPLC gradient consisted of mobile phases: A (0.1 mM sodium acetate, pH 7.2) and B (methanol) as follows: 0–1 min, 3% B; 1.1–6.5 min, 14% B; 6.6–9.0 min, 100% B; and 9.1–16 min, 3% B. The analysis and combination of chromatographic peaks was generated in Millenium-32 Software (Waters, Milford, MA).

#### *RNA Isolation Procedure*

Total cellular RNA was isolated from endometrial tissue samples using Trizol reagent (Invitrogen, cat. no. 15596-026, Carlsbad, CA) according to manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively. For the *in vitro*

study, RNA from each flask was extracted using an RNeasy Mini kit (Qiagen, cat.no. 74104, Valencia, CA) following manufacturer's protocols for amplification and RT-PCR.

### *In Situ Hybridization Analyses*

Localization of *SLC5A1*, *SLC7A1*, *SLC7A2*, and *SLC7A3* mRNAs in sections (5µm) of swine uteri was determined by radioactive *in situ* hybridization analyses as described previously (Johnson, et al. 1999b). Radiolabeled antisense or sense cRNA probes were generated by *in vitro* transcription using linearized plasmid template, RNA polymerases, and [ $\alpha$ -35S]-UTP. Deparaffinized, rehydrated and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes. After hybridization, washing and ribonuclease A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak, Rochester, NY), and exposed at 4°C for seven to ten days. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher Scientific, Fairlawn, NJ). Digital images of representative fields were recorded under brightfield or darkfield illumination and evaluated using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera and Axiovision 4.3 software. Photographic plates were assembled using Adobe Photoshop (version 6.0, Adobe Systems Inc., San Jose, CA).

### *RT-PCR and Quantitative PCR Analyses*

Partial mRNAs (3µg) for swine endometrial *SLC5A1* and *SLC7A3* were amplified by RT-PCR using tRNA from endometria collected from uteri of gilts hysterectomized on Day 12 and 15 of pregnancy or from placentae of gilts hysterectomized on day 30 of pregnancy, respectively. For *SLC5A1*, the sense primer (5'-taccagcctcttgggctcta-3') and antisense primer (3'-acaaccacccaaatcagagc-5') were derived from the swine *SLC5A1* mRNA coding sequence. For *SLC7A3*, the sense primer (5'-cactcaactccatccccact-3') and antisense primer (3'-ctgtggctgtctccagatga-5') were derived from the porcine *SLC7A3* mRNA coding sequence (All primers were ordered from Invitrogen, San Diego, CA.). Porcine *SLC5A1* (152 bp) and swine *SLC7A3* (206 bp) products were amplified along with the same amount of beta-actin controls using a SuperScript® III Reverse Transcriptase Kit (Invitrogen, San Diego, CA). The PCR amplification procedures were : 1) 95°C for 2 min; 2) 58°C for 30 sec, 50°C for 45 sec, and 72°C for 1 min for 25 cycles; and 3) 72°C or 7 min. All samples were loaded in 2% agarose gels containing Bio-Safe (Bio-Rad, Hercules, CA) for electrophoresis at 100 V for 1. Images were captured by UVP PhotoDoc-It™ Imaging Systems fitted with a Canon A480 digital camera in benchtop UV transilluminator (UVP, LLC Upland, CA), followed by densitometry and statistical analyses. Quantitative real-time PCR was performed on a Roche LightCycler 480 real-time PCR system (Roche, USA) with Perfecta SYBR Green Fastmix (Quanta Biosciences, Gaithersburg, MD) and 2.5 µM forward/reverse primers to determine transcript levels in three replicates (each measured in triplicate). Conditions for cycles were as follows: 95°C for 5 min, 40 cycles of 95°C for 10 sec and 60°C for 30 sec. To

determine primer efficiency, a relative standard curve was analyzed using a dilution series of the appropriate cDNA. Data were analyzed using the comparative Ct method, normalized to the endogenous control gene *GAPDH* (endometrial mRNA) or the geometric mean of *TBP/SDHA/TUBA1B* (placental mRNA).

#### *Measurement of Transport of Arginine by Placenta*

Arginine transport in pig placenta was determined using L-[U-<sup>14</sup>C] arginine (American Radiolabeled Chemicals (St. Louis, MO) as described previously for branched-chain amino acids (Self, et al. 2004). Samples of placenta (~200 mg) were washed three times in oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>, v/v) Krebs-Henseleit bicarbonate (KHB) buffer containing 20 mM HEPES (pH 7.4) and 5 mM glucose. Samples were then incubated at 37°C for 5 min in 1 ml of oxygenated KHB buffer consisting of 20 mM HEPES, 2 mM glutamate, 5 mM glucose, 0.5 or 2 mM arginine, 0.05 µCi L-[U-<sup>14</sup>C] arginine, and 0.05 µCi [<sup>3</sup>H] inulin (an extracellular marker). After the 5-min incubation, the tissues were rinsed thoroughly with fresh KHB buffer and then solubilized in 0.5 ml of Soluene 350. The solution was measured for <sup>14</sup>C and <sup>3</sup>H radioactivities using a dual-channel counting program in a Packard 1900 liquid scintillation counter (Meriden, CT). The specific activity of [<sup>14</sup>C] arginine in the medium was used to calculate arginine uptake by placenta. Results from preliminary experiments established that arginine uptake was linear over a 5-min period.

### *Statistical Analyses*

Densitometry analyses were converted by ImageJ (NIH, Bethesda, MD). Data were processed in analysis of variance (ANOVA) and student's T-test. Densitometry values were standardized to sample loading control using GraphPad Prism (GraphPad Software, San Diego, CA). Partial data are presented with standard errors (SE). Tests of statistical significance were generated using the appropriate error terms according to the expectation of mean squares. Statistical analyses for qPCR were run using a one-way ANOVA with Tukey's test for mean comparison.

## **Results**

### *Glucose and Amino Acids in Uterine Flushings*

Glucose increased in the uterine flushings from both cyclic and pregnant gilts between Days 10 and 13 ( $P<0.05$ ) (Fig. 3.1). Glucose was not different in uterine flushings from cyclic and pregnant gilts ( $P>0.05$ ).

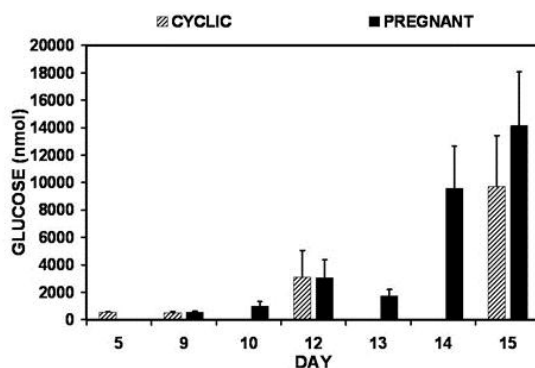


Fig. 3.1. Glucose increased in uterine flushings of cyclic and pregnant gilts.

Arginine was increased in the uterine flushings of Day 15 pregnant over Day 15 cyclic gilts ( $P<0.05$ ) (Fig. 3.2). No differences in the quantity of leucine and glutamine were detected within the uterine flushings of cyclic and pregnant gilts ( $P>0.05$ ) (Fig. 3.2).

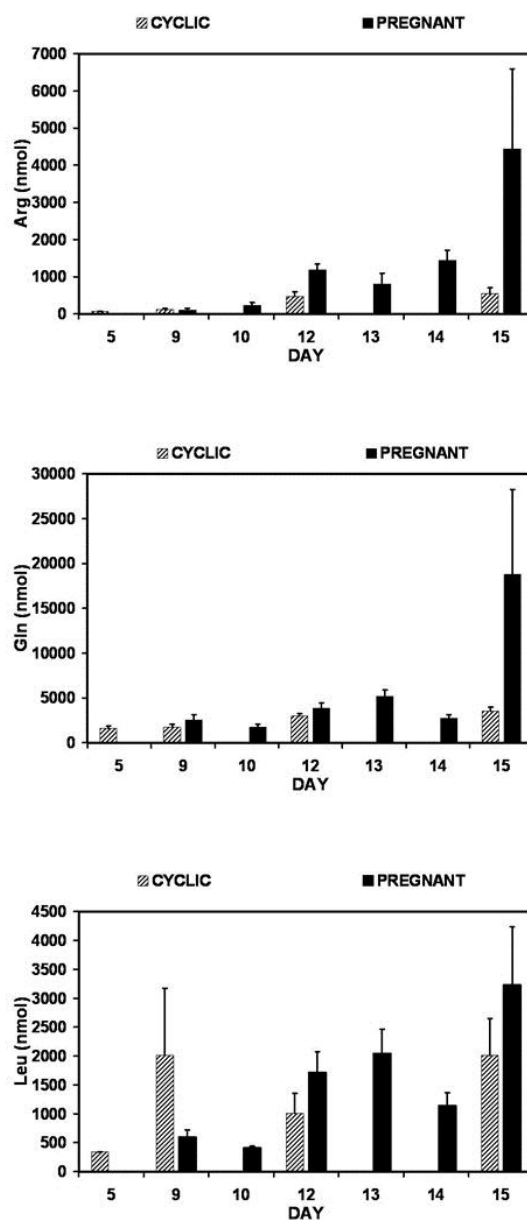


Fig. 3.2. Arg, Leu and Glu in uterine flushings of cyclic and pregnant gilts.

### *SLC5A1 mRNA Expression in Uteri and Placentae*

*In situ* hybridization localized *SLC5A1* mRNA to the endometrial LE and GE of both cyclic and pregnant gilts (Fig. 3.3). *SLC5A1* was induced in both the LE and GE of both cyclic and pregnant gilts between Days 5 and 9, and then decreased in these cell types between Days 9 and 12 of the estrous cycle (Fig. 3.3). In contrast to the estrous cycle, *SLC5A1* mRNA further increased in the LE between Days 9 and 12 and mRNA reached maximal levels in LE by Day 13 (Fig. 3.3). *SLC5A1* then decreased to background levels in all cell types (both endometrial and placental) between Days 13 and 14 of gestation (Fig. 3.3). RT-PCR and qPCR confirmed that *SLC5A1* mRNA levels are higher in the endometrium of Day 12 pregnant over Day 12 cyclic gilts (Fig. 3.4).

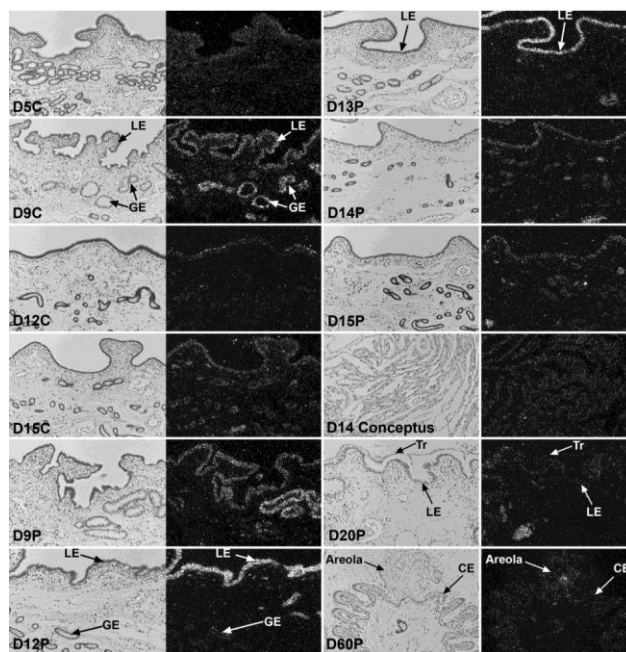


Fig. 3.3. *In situ* hybridization for *SLC5A1* expression in the endometria of cyclic and pregnant gilts. *SLC5A1* mRNA increases initially in the luminal epithelium (LE) and GE of both cyclic and pregnant gilts, but mRNA expression further increases in the LE of pregnant gilts by Day 12.



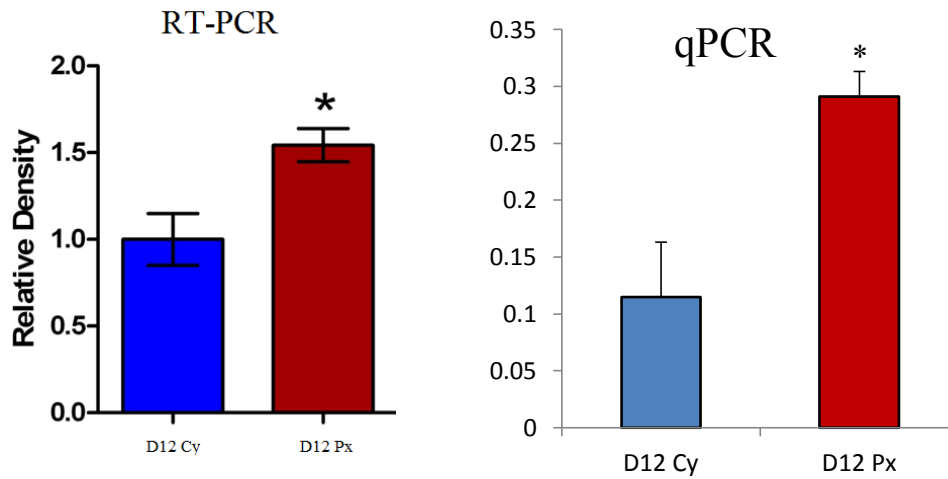


Fig. 3.4. RT-PCR and qPCR for *SLC5A1* mRNA in endometrium of Day 12 cyclic and pregnant gilts. Steady state levels of *SLC5A1* mRNA are greater in the endometria of Day 12 pregnant over cyclic gilts (Normalization factor for rt-PCR is *beta-actin* and for qPCR is *GAPDH*) (\* =  $P < 0.05$  or statistically significant difference).

#### *Estrogen Upregulates SLC5A1 Expression in Endometrium*

*In situ* hybridization localized *SLC5A1* mRNA to the endometrial LE of Day 15 pseudopregnant gilts suggesting estrogen specifically induces expression of *SLC5A1* mRNA in the LE (Fig. 3.5). In agreement with *in situ* hybridization data, steady state levels of *SLC5A1* mRNA as measured by RT-PCR and qPCR increased in the endometrium of estrogen-treated (pseudopregnant) over CO-treated cyclic gilts (Fig. 3.6). Treatment of cultured porcine LE cells confirmed that estrogen can directly increase the *SLC5A1* transcription in the LE (Fig. 3.7).

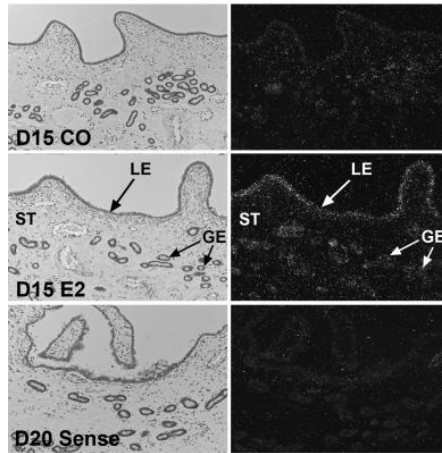


Fig. 3.5. *In situ* hybridization for *SLC5A1* mRNA in the endometria of Day 15 cyclic (CO) and pseudopregnant (E2) gilts. Estrogen induces expression of *SLC5A1* mRNA in the LE of pseudopregnant gilts.

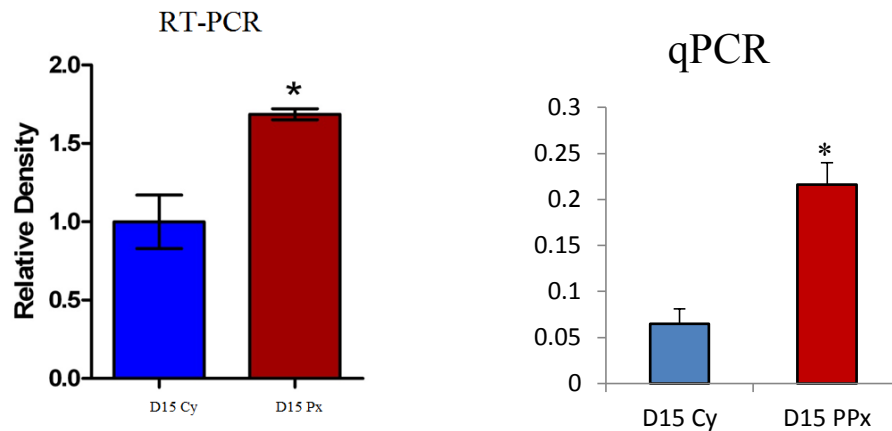


Fig. 3.6. RT-PCR and qPCR for *SLC5A1* mRNA in the endometria of Day 15 cyclic and pseudopregnant gilts. Steady state levels of *SLC5A1* mRNA are greater in endometria of pseudopregnant over cyclic gilts (Normalization factor for rt-PCR is *beta-actin* and for qPCR is *GAPDH*) (\* =  $P < 0.05$  or statistically significant difference)

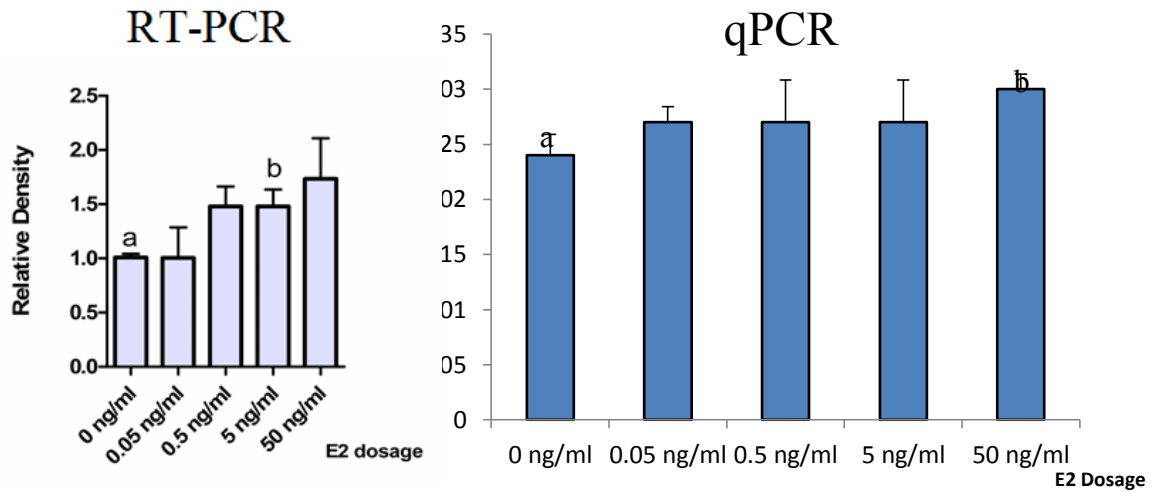


Fig. 3.7. RT-PCR and qPCR for the *in vitro* expression of *SLC5A1* mRNA in porcine endometrial LE cells treated with estrogen. Estrogen dose-dependently increased the expression of *SLC5A1* mRNA in porcine LE cells (Normalization factor for rt-PCR is *beta-actin* and for qPCR is *GAPDH*) (a and b has statistically significant difference or  $p < 0.05$ ).

#### *Amino Acid Transporter SLC7A3 Increase in Later Pregnancy in Gilts*

*In situ* hybridization of cationic amino acid transporter *SLC7A3* mRNA shows that *SLC7A3* mRNA expression increases in the trophoblast/chorion between Days 20 and 25 then reaches maximal levels by Day 30 (Fig. 3.7). No expression of *SLC7A1* and *SLC7A2* were detected. Strong expression of *SLC7A3* persists in areolae and interareolar region through Day 85 of pregnancy (Fig 3.8). *SLC7A3* mRNA increases in placentae between Days 30 and 60 of pregnancy ( $P < 0.05$ ) (Fig 3.9). No significant difference in the arginine transport was found between areolae and inter-areolae area across the placentae of pregnant gilts ( $P > 0.05$ ) (Fig 3.10).

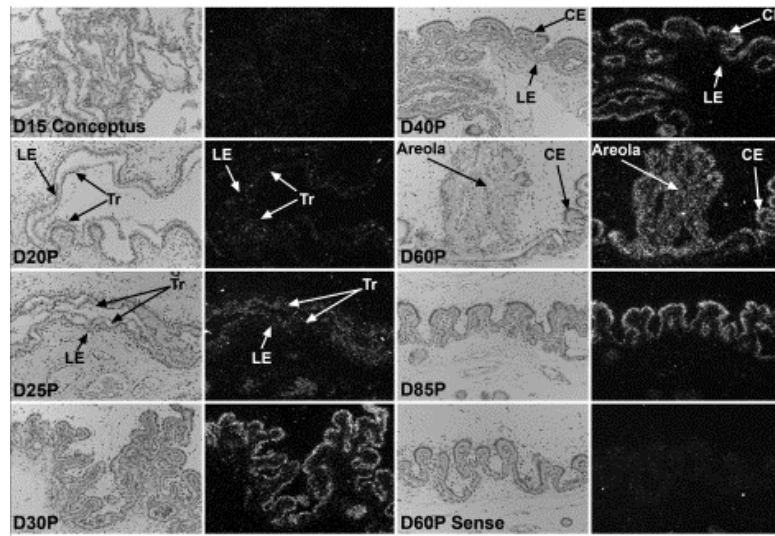


Fig. 3.8. *In situ* hybridization for *SLC7A3* expression in the placentae of gilts. *SLC7A3* mRNA increases in trophoblast/chorion between Days 20 and 25, increases to maximal levels by Day 30, and high expression of *SLC7A3* is maintained in areolae and interareolar trophoblast/chorion through Day 85 of pregnancy.

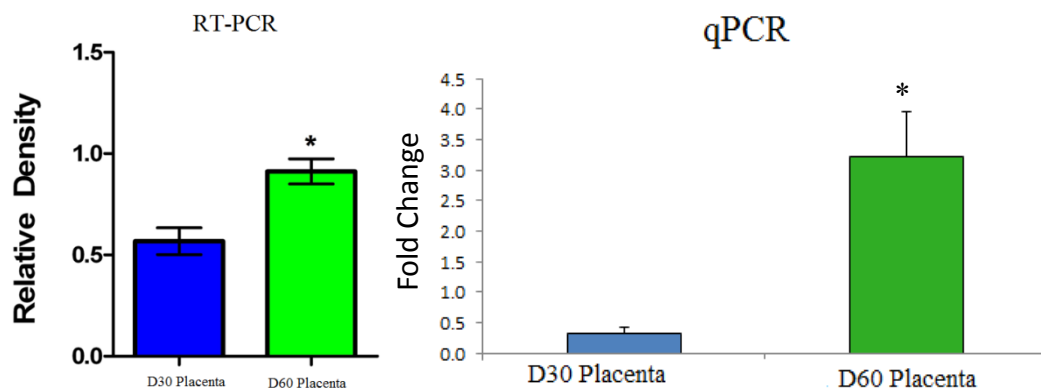


Fig. 3.9. RT-PCR and qPCR results for *SLC 7A3* mRNA in placenta of Day 30 and Day 60 pregnant gilts. *SLC7A3* mRNA increases in placentae between Days 30 and 60 of pregnancy (Normalization factor for rt-PCR is *beta-actin* and for qPCR is geometric mean of *TBP/SDHA/TUBA1B*) (\* =  $P < 0.05$  or statistically significant difference).

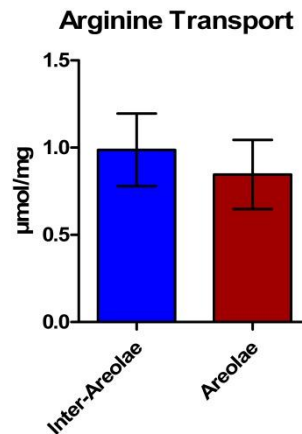


Fig. 3.10. Arginine transport in areolae and inter-areolae area across the placentae of Day 60 pregnant gilts. No significant difference in the transport of arginine was found between areolae and inter-areolae across the placentae of Day 60 pregnant gilts ( $P>0.05$ ).

## Discussion

Pig implantation and placentation are different from that in sheep because porcine have an epitheliochorial placenta in which uterine luminal epithelium is superficial but remain intact throughout pregnancy. Pig trophoctoderm attaches to the luminal epithelium and serves as the platform for maternal hematotropic and histotropic nutrient support for conceptus growth (Björkman 1973, Corner 1921). Pig implantation is a fascinating model to investigate and placentation-associated gene regulation is very unique. Estrogen is secreted by pig conceptuses between Days 10 and 15 for pregnancy recognition, which redirects prostaglandin F<sub>2</sub> secretion from an endocrine (toward uterine vasculature) to an exocrine (toward the uterine lumen) pattern and later metabolized to prevent luteolysis of the corpus luteum (Bazer 1983). The importance of

estrogen in pig pregnancy has been studied extensively. E2 increase expression of numerous genes within the uterine LE such as *FGF7*, *AKR1B1*, *IRF2*, *MX1*, *EDG7*, *NMB*, *B2M* and *CD24* to enhance proliferation, migration, adhesion and gene expression that supports implantation and conceptus development (Bazer, et al. 2012b)

The importance of glucose and amino acid in pigs has been underscored previously. Arginine supplement to the diet of pregnant gilts between days 30 and 114 of pregnancy significantly increased litter birth-weight and the number of live-born piglets (Wu, et al. 2007). In this study, we measure the glucose levels in uterine flushing in gilts and found the transitory increase at day 12 of pregnant gilts. The level of glucose in uterine flushings could not be explained by facilitated glucose transporter expressions patterns such as *SLC2A1-3* (unpublished results by Johnson et al.). We hypothesized that sodium dependent glucose transporter *SLC5A1* plays an important role in this sudden increase in the uterine flushings. Since this is also a critical time period in swine pregnancy when estrogen, the pregnancy recognition signal is secreted, may regulate the expression of this gene. A pseudopregnant model was utilized for *in situ* hybridization. *SLC5A1* expression was remarkably higher in the estrogen-treated gilts. An *in vitro* experiment also shows expression of *SLC5A1* increases dose-dependently with estrogen treatment.

The arginine family of amino acid plays an important role in swine reproduction and promote nutrient metabolism, such as glucose synthesis because arginine, glutamine, glutamate, proline, aspartate, asparagine, ornithine, and citrulline are interconvertible via complex interorgan metabolism (Wu and Meininger 2002). The cationic amino acid transporters (CATs) comprise the *SLC7* family and *SLC7A1-3* are known plasma

membrane transporters that accept arginine as a substrate (Closs, et al. 2004). Arginine plays a key role in placental angiogenesis and growth in mammals; and arginine provision enhances reproductive performance of gilts (Mateo, et al. 2007). The temporal and spatial expression of cationic amino acid transporter *SLC7A1* was demonstrated in this study. Our *in situ* hybridization results suggest that *SLC7A1* mRNA expression significantly increases from Day 30 to Day 60 in the trophoblast/chorion through Day 85 of pregnancy and support an interpretation that *SLC7A1* plays a key role in carrying essential amino acid across the placenta to maintain nutrients transport and support fetal growth in mid to late pregnancy in gilts. This increase in the *SLC7A3* expression corresponds with previous data published by Wu et al. showing the increase transport of arginine in pigs (Wu, et al. 1995). No significant difference in the transport of arginine was found between areolae and inter-areolae across the placenta of Day 60 pregnant gilts. Paracellular transport may be upregulated at this time due to the change in expression pattern of tight junction proteins including claudins (unpublished results by Johnson et al.).

## CHAPTER IV

### CONCLUSIONS

Nutrients provided by the maternal and placental vasculatures during healthy pregnancy, including glucose and amino acids, are known to play a critical role in optimizing embryonic growth and regulating fetal development (Bloomfield, et al. 2013a). Uterine nutrients are supplied via the uterine vasculature and in pigs that have true epitheliochorial placentation, nutrient are transported across the uterine luminal epithelium (LE) and placental chorion to gain access to the placental allantoic vasculature that serves to transport these nutrients, rich in sugars, amino acids, proteins and electrolytes to the fetus (Bazer, et al. 2012a, Wu, et al. 2010).

Placental development is highly dependent on the growth of new blood vessels (neovascularization) during pregnancy in all forms of placentation including hemochorial placentation in humans, mice, and rats, endotheliochorial placentation in most carnivores such as cats and dogs, synepitheliochorial placentation in ruminants such as sheep and cows, and epitheliochorial placentation of pigs, horses, camels and whales. One important cell type involved in the neoangiogenesis is the endothelial EPC which originates from the bone marrow and enters the systemic circulation for distribution to sites of neoangionenesis. This monocytic cell has the ability to differentiate into endothelial cells and contribute to vascular sprouting.

OPN, a matricellular protein that is highly upregulated during pregnancy, is abundantly expressed in the extracellular matrices of the uterine and placental stroma of



pigs (Garlow, et al. 2002, White, et al. 2005). This expression is highly correlated to sites of angiogenesis. Many characteristics of OPN and EPCs are complimentary to each other: (1) EPCs express integrins while OPN binds to integrins; (2) EPCs home to sites of inflammation while OPN is upregulated during inflammation; (3) EPCs differentiate into endothelial cells while OPN is an endothelial cell chemoattractant; (4) EPCs are involved in pathologic angiogenesis while OPN is upregulated in these pathology; and (5) EPCs, increase during healthy pregnancy while OPN is highly upregulated during gestation (Apte, et al. 2005, Asahara 1997, Asou, et al. 2001, Bailey, et al. 2010, Buemi, et al. 2007, Caiado and Dias 2012, Denhardt and Guo 1993, Duvall, et al. 2007, Erikson, et al. 2009, Garlow, et al. 2002, Gomer 2008, Hristov, et al. 2003, Matsubara, et al. 2006, White, et al. 2005). The relationship between EPCs and OPN in uterine and placental tissues has not been previously investigated.

The first part of this thesis identified and determined the physiological pathways that promote vascular growth within the placenta *in vitro*. Porcine EPCs were isolated from newborn piglets and propagated in culture for more than 30 passages with stocks of cells frozen back at each passage. These EPCs developed an endothelial-like phenotype in culture as endothelial cell markers increased while monocyte markers decreased with increasing passage. EPCs express integrins including ITGAV, and use these transmembrane receptors to adhere and migrate *in vitro* on osteopontin. An innovative three-dimensional collagen matrix model supplemented with potent angiogenesis promoting S1P was used to explore functional characteristics of EPCs, and their surface

markers, that allow EPCs to form angiogenic networks in three-dimensions and incorporate into vascular networks.

A novel discovery in this investigation was that OPN increased recruitment of circulating EPC to a developing vascular network. When EPCs were cultured alone as a monolayer on the surface of 3D collagen matrices supplemented with angiogenic factors, they failed to invade the matrix; however, when cultured with HUVECs, which invade the matrix and form vascular structures, the EPCs incorporate into these vascular structures, similar to their role *in vivo*. Furthermore, the number of EPCs that invaded the matrix increased in a dose-dependent manner in the presence of OPN.

OPN contains several functional domains including binding sites for ITGAV (the (Caiado and Dias 2012, Casals, et al. 2010). Porcine EPCs were shown to express the ITGAV which has been shown to bind OPN (Fig. 2.2b, Fig. 2.3a). Silencing the function of the *ITGAV* gene by siRNA decreased the ability of EPCs to bind OPN and decreased ITGAV-associated focal adhesions including the focal adhesion vinculin and paxillin involved in transducing force between cells and the ECM necessary to promote invasion. *ITGAV* silencing also blocked the ability to incorporate into actively invading HUVECs. These investigations have allowed the development of a novel theory of how placental vascularization maximizes during pregnancy. This includes recruitment of EPCs from the circulation by OPN and their incorporation into growing vessels.

The specific junctional proteins responsible for the EPC-HUVEC crosstalk remain to be determined. Adherens, gap, and tight junctions play a crucial role in angiogenic responses (Gärtner, et al. 2012, Wallez and Huber 2008, Wallez, et al. 2006,

Yu, et al. 2011) and may be involved in HUVEC directed EPC incorporation into angiogenic network. Based upon previous studies that suggest that soluble factors secreted by HUVECs do not stimulate EPC invasion and therefore the mechanisms involving EPC incorporation into vasculature are expected to be a result of direct physical communication between EPCs and HUVECs. Immediate future studies will investigate the role of junctional communication between HUVECs and EPCs. Specific endothelial cadherins, occludins and gap junctions proteins have been selected in future studies as targets for knockdown experiments.

While our understanding of placental and uterine angiogenesis, and the role of EPCs in this process, is incomplete and presents a barrier to development of new therapies to alleviate or prevent prenatal defects. EPCs could become a powerful therapeutic tool because of its mobility, differential potential and possibility to inject intravenously. These investigations contribute to a more mechanistic understanding of the behavior of EPCs and may contribute to the development of novel strategies targeting diseases in pregnancy associated with dysregulated angiogenesis.

In pigs, glucose can be transported from both sides of placenta. Two families of cellular glucose transporters have been identified: the facilitated-diffusion glucose transporters (GLUT) and the sodium-dependent glucose transporters (SGLT). Many glucose transporters and most amino acid transporters belong to the solute carrier family (*SLC*). The SGLTs, which includes *SLC5A* and cationic amino acid transporters, which belongs to the *SLC7* family, are known to be expressed in mammalian species (Bell, et al. 1990, Fotiadis, et al. 2013, Gao, et al. 2012). However, while glucose and amino acid

transporter expression patterns have been identified in some species, the regulated expression glucose and amino acid transporters in uterine and placental tissues during pregnancy has not been fully established in pigs.

The second part of this thesis determined temporal and spatial cell-specific changes in expression of *SLC5A1* and *SLC7A3* in porcine conceptus and uteri during the estrous cycle and during the peri-implantation period of pregnancy and the effect of estrogen on their expression in the uterus. Glucose and amino acid (arginine) transport level increased in uterine flushings in pregnant over cyclic gilts at the periimplantation period. Notably, the increase in glucose level at day 12 in pregnancy gilts correlated with upregulated *SLC5A1* expression in quantitative PCR analysis. The mechanism for this upregulation was shown to involve estrogen, the pregnancy recognition signal in pigs, based upon *in vivo* and *in vitro* experiments. These studies also provide new insights for the basis of the observation that arginine levels increase in the placenta during late pregnancy in the pig. The increase in arginine correlated with the expression of a major cationic amino acid transporter, *SLC7A3*, which increased later pregnancy in gilts.

This investigation provides new insights into the expression pattern and regulation of glucose and amino acid transporters in porcine pregnancy. It is hoped that this fundamental knowledge can provide a more comprehensive knowledge of nutrient transport to the fetus that may be useful in the development of clinical strategies to reduce infertility and prevent pregnancy loss in domestic livestock where the majority of embryonic loss occurs primarily at peri-implantation period of early pregnancy (Wilmut, et al. 1986, Wu, et al. 2010).

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